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The Human Pancreatic Islet Methylome and Its Role in Type 2 Diabetes

TASNIM DAYEH

DEPARTMENT OF CLINICAL SCIENCES | LUND UNIVERSITY 2016



The Human Pancreatic Islet Methylome and Its Role in Type 2 Diabetes

Tasnim Dayeh



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DOCTORAL DISSERTATION

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To be defended at the Jubilee Auditorium, on February 3rd 2016, at 9.00

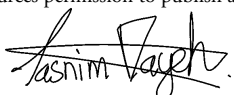
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<p>Abstract</p> <p>Islet dysfunction is central to the development and progression of type 2 diabetes (T2D). Epigenetic modifications are essential for establishing and maintaining cell identity and function in normal circumstances. Exposure to adverse environmental factors may alter the epigenome, and result in changes of gene expression and the resulting phenotype. The aim of this thesis was to analyze DNA methylation levels of specific genes, as well as genome-wide DNA methylation, in order to determine whether epigenetic dysregulation of pancreatic islets contributes to islet dysfunction in subjects with T2D. We also assessed the relationship between genetic variation and DNA methylation. We further examined the potential use of DNA methylation in blood DNA to predict future T2D.</p> <p>At the specific gene level, we found that DNA methylation of <i>INS</i> and <i>PDX-1</i> was increased in pancreatic islets from subjects with T2D (Studies I and II). Conversely, their mRNA expression, insulin content and glucose-stimulated insulin secretion (GSIS) were decreased in the same islets. We next analyzed genome-wide DNA methylation in human pancreatic islets from both T2D and non-diabetic donors (Study III). Nearly 1,500 CpG sites (853 genes) were differentially methylated in T2D islets, with the majority showing decreased DNA methylation. 102 genes showed both altered DNA methylation and mRNA expression in T2D islets, including <i>CDKN1A</i>, <i>PDE7B</i>, <i>SEPT9</i> and <i>EXOC3L2</i>. Our functional experiments provided further evidence that altering the expression of these genes, by modeling the situation in T2D, results in impaired insulin and glucagon secretion in cell line models.</p> <p>Furthermore, we showed that nearly half of the single nucleotide polymorphisms (SNPs) associated with T2D are CpG-SNPs, which can introduce or remove a CpG site (Study IV). Accordingly, we found that the degree of DNA methylation at CpG-SNP sites varied between individuals with different genotypes, and that some of the CpG-SNPs were associated with differential gene expression, alternative splicing and hormonal secretion.</p> <p>In Study V, we showed that altered DNA methylation at two CpG sites in the <i>ABCG1</i> and <i>PHOSPHO1</i> genes in blood from non-diabetic individuals was associated with a higher risk of future T2D. Subsequently, we found that CpG sites annotated to these genes were differentially methylated in T2D target tissues.</p> <p>Taken together, our findings suggest that epigenetic dysregulation of pancreatic islets play a role in islet dysfunction in subjects with T2D, and can be influenced by genetic variation and the environment.</p>		
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The Human Pancreatic Islet Methylome and Its Role in Type 2 Diabetes

Tasnim Dayeh



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Experiment.

Fail.

Learn.

Repeat.

∞

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List of publications

Scientific papers contributing to this thesis

- I. Yang BT, Dayeh TA, Kirkpatrick CL, Taneera J, Kumar R, Groop L, Wollheim CB, Nitert MD, Ling C: *Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA(1c) levels in human pancreatic islets*. Diabetologia 2011, 54:360-367.
- II. Yang BT, Dayeh TA, Volkov PA, Kirkpatrick CL, Malmgren S, Jing X, Renstrom E, Wollheim CB, Nitert MD, Ling C: *Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes*. Mol Endocrinol 2012, 26:1203-1212.
- III. Dayeh T, Volkov P, Salo S, Hall E, Nilsson E, Olsson AH, Kirkpatrick CL, Wollheim CB, Eliasson L, Ronn T, et al: *Genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors identifies candidate genes that influence insulin secretion*. PLoS Genet 2014, 10:e1004160.
- IV. Dayeh TA, Olsson AH, Volkov P, Almgren P, Ronn T, Ling C: *Identification of CpG-SNPs associated with type 2 diabetes and differential DNA methylation in human pancreatic islets*. Diabetologia 2013, 56:1036-1046.
- V. Dayeh T, Tuomi T, Almgren P, Perfilyev A, Jansson PA, Pihlajamäki J, De Mello V, Vaag A, Groop L, Nilsson E and Ling C: *DNA methylation at ABCG1 and PHOSHO1 in blood DNA is associated with future type 2 diabetes risk*. (Submitted to Epigenetics)

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Prokopenko I, Poon W, Magi R, Prasad BR, Salehi SA, Almgren P, Osmark P, Bouatia-Naji N, Wierup N, Fall T, et al: *A central role for GRB10 in regulation of islet function in man*. PLoS Genet 2014, 10:e1004235.

Olsson AH, Volkov P, Bacos K, Dayeh T, Hall E, Nilsson EA, Ladenvall C, Ronn T, Ling C: *Genome-wide associations between genetic and epigenetic variation influence mRNA expression and insulin secretion in human pancreatic islets*. PLoS Genet 2014, 10:e1004735.

Hall E, Volkov P, Dayeh T, Bacos K, Ronn T, Nitert MD, Ling C: *Effects of palmitate on genome-wide mRNA expression and DNA methylation patterns in human pancreatic islets*. BMC Med 2014, 12:103.

Hall E, Volkov P, Dayeh T, Esguerra JL, Salo S, Eliasson L, Ronn T, Bacos K, Ling C: *Sex differences in the genome-wide DNA methylation pattern and impact on gene expression, microRNA levels and insulin secretion in human pancreatic islets*. Genome Biol 2014, 15:522.

Hall E, Dayeh T, Kirkpatrick CL, Wollheim CB, Dekker Nitert M, Ling C: *DNA methylation of the glucagon-like peptide 1 receptor (GLP1R) in human pancreatic islets*. BMC Med Genet 2013, 14:76.

Ronn T, Volkov P, Davegardh C, Dayeh T, Hall E, Olsson AH, Nilsson E, Tornberg A, Dekker Nitert M, Eriksson KF, et al: *A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue*. PLoS Genet 2013, 9:e1003572.

Nitert MD, Dayeh T, Volkov P, Elgzyri T, Hall E, Nilsson E, Yang BT, Lang S, Parikh H, Wessman Y, et al: *Impact of an exercise intervention on DNA methylation in skeletal muscle from first-degree relatives of patients with type 2 diabetes*. Diabetes 2012, 61:3322-3332.

Dayeh T, Ling C: *Does epigenetic dysregulation of pancreatic islets contribute to impaired insulin secretion and type 2 diabetes?* Biochem Cell Biol 2015:1-11.

Abbreviations

2hrPG	2 hour plasma glucose
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BCA	bicinchoninic acid assay
BMI	Body mass index
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CI	confidence interval
CpG	cytosine guanine dinucleotide
Ct	cycle threshold
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dsRNA	double stranded RNA
DTZ	dithizone
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FDR	false discovery rate
FPG	fasting plasma glucose
GLP-1	glucagon-like peptide-1
GSIS	glucose-stimulated insulin secretion
HA	hemagglutinin
HbA1c	glycated hemoglobin
HBSS	HEPES balanced salt solution
HDAC	histone deacetylase
HDL	High-density lipoprotein
HOMA-IR	Homeostasis Model Assessment-estimated Insulin Resistance
HPLC	high-performance liquid chromatography
HTL	human tissue lab
IE	islet equivalent

KOBS	Kuopio obesity surgery study
LD	linkage disequilibrium
LUDC	Lund University Diabetes Center
MAF	minor allele frequency
MALDI TOF MS	matrix-assisted laser desorption/ionization time of flight mass spectrometry
MIDY	Mutant INS-gene-induced Diabetes of Youth
mRNA	messenger RNA
MZ	monozygotic
N shelf	North shelf
N shore	North shore
NASH	nonalcoholic steatohepatitis
nm	nanometer
OGTT	oral glucose tolerance test
OR	odds ratio
PCA	principal component analysis
PCR	polymerase chain reaction
RIA	radioimmunoassay
RISC	RNA-induced silencing complex
RMA	Robust Multi-array Average
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RT	reverse transcription
S shelf	South shelf
S shore	South shore
SAP	shrimp alkaline phosphatase
SAM	S-adenosylmethionine
SD	standard deviation
SEM	standard error of mean
SI	stimulation index
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SSC	side scatter
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TET	ten-eleven translocation
TF	transcription factor
TSS	transcription start site
UTR	untranslated region

Introduction

Type 2 diabetes (T2D) is a metabolic disorder driven by an excess in energy that manifests itself in the form of chronic high blood glucose levels. T2D develops when β -cells are unable to secrete adequate amounts of insulin to compensate for increasing demands in response to insulin resistance in genetically and epigenetically susceptible people. Eventually, chronic hyperglycemia may lead to severe tissue damage in vulnerable organs, and to secondary complications. Although a non-communicable disease, the growing incidence of T2D worldwide has turned it into a pandemic, placing an enormous burden on the affected individual, and on society [1, 2].

Pancreatic Islet Dysfunction in Type 2 Diabetes

The balance between glucose production and utilization is known as glucose homeostasis. In healthy individuals, this balance is maintained by a number of regulatory hormones that work together, including insulin and glucagon [3]. During the fasting state, glucagon secretion from pancreatic α -cells stimulates glucose release from the liver into the blood (Figure 1A). Glucose is used by a number of tissues, including the brain which is exclusively dependent on glucose. In contrast, other tissues, such as skeletal muscle and the heart, metabolize non-glucose nutrients (for example, non-esterified fatty acids generated from adipose tissue lipolysis). During the fasting state, pancreatic islets maintain a low insulin to glucagon ratio in order to prevent hypoglycemia. Following a meal, glucose is absorbed by the gut, and as a result blood glucose levels increase (Figure 1B). Pancreatic islets sense the increase in glucose, and respond by secreting more insulin and less glucagon. In addition, glucagon-like peptide-1 (GLP-1) secreted from the gut enhances glucose-stimulated insulin secretion (GSIS), and suppresses glucagon secretion, which further increases the insulin to glucagon ratio. Insulin also prompts glucose uptake and storage by skeletal muscle and adipose tissue, and suppresses lipolysis from adipose tissue and hepatic glucose production [1]. When target tissues are no longer responsive to insulin signaling, a state known as insulin resistance, the pancreatic β -cells attempt to compensate by secreting more and more insulin until the point at which they cannot compensate any longer. Failure to take up and store excess glucose results in hyperglycemia. T2D is diagnosed by a chronic increase in blood glucose levels in the fasting and/or fed state.

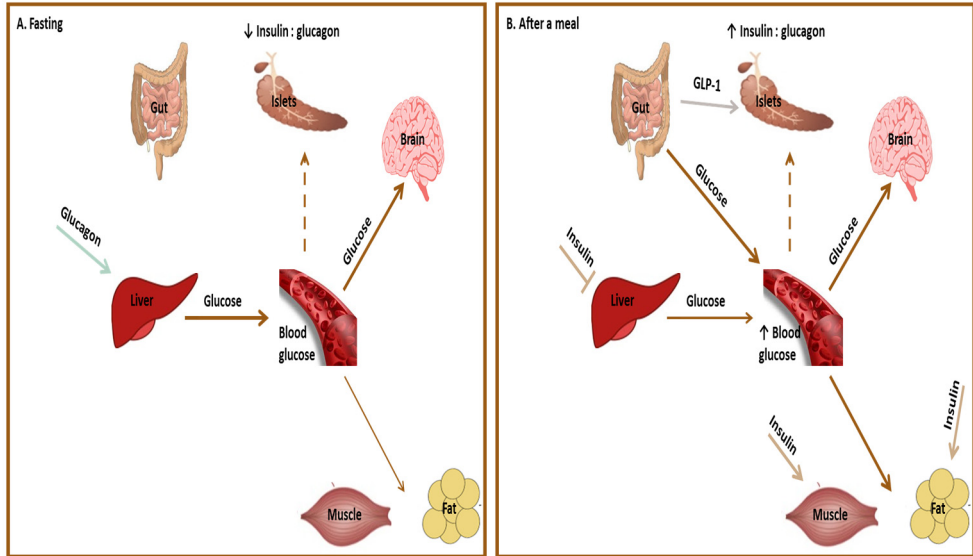


Figure 1. Glucose homeostasis.

Picture is adapted from [1].

Pancreatic β -cells play a central role in the development and progression of T2D [4]. T2D only develops when β -cells fail to compensate for the increase in demand for insulin [5]. The progressive loss of β -cell function in T2D goes through a number of different and partially reversible stages, each of which is characterized by changes in β -cell mass, function and phenotype [6]. β -cell dysfunction may be precipitated by a number of pathological stressors, including: endoplasmic reticulum (ER) stress, metabolic and oxidative stress, amyloid plaques, inflammation, and disruption of islet integrity (Figure 2) [4]. β -cells continue to be one of the main targets for T2D therapy. Treatment strategies that aim to preserve β -cell function by reducing its workload, such as the use of the drug metformin, are considered to provide the most effective diabetes treatments [5]. Additionally, ongoing attempts are being made to regenerate functional β -cells from stem cells, or non- β islet cells [7, 8].

Although T2D was initially thought to be a bi-hormonal disease, the role of glucagon-secreting α -cells has been neglected until recently [9, 10]. Glucagon is a counter-regulatory hormone to insulin that elevates blood glucose levels during hypoglycemia by stimulating gluconeogenesis and glycogenolysis from liver hepatocytes. Pancreatic α -cells and β -cells regulate each other, and through their partnership normoglycemia is maintained [9, 11]. In subjects with T2D, there is an increase in glucagon secretion when glucose levels are high, which exacerbates already existing hyperglycemia; but inadequate glucagon is produced when glucose levels are low, which can cause hypoglycemia [12]. Interestingly, it has been shown that glucagon receptor-null mice that were exposed to β -cell destruction by streptozocin,

remained clinically normal without hyperglycemia, thus suggesting that metabolic manifestations of insulin deficiency did not occur without the action of glucagon [13]. Glucagon suppression is an essential part of the GLP-1 effects on lowering hyperglycemia [9]. Thus α -cells are valuable targets for new drugs. Controlling hyperglucagonemia by blocking glucagon receptors, or by suppressing glucagon secretion, may help to reduce hyperglycemia [9].

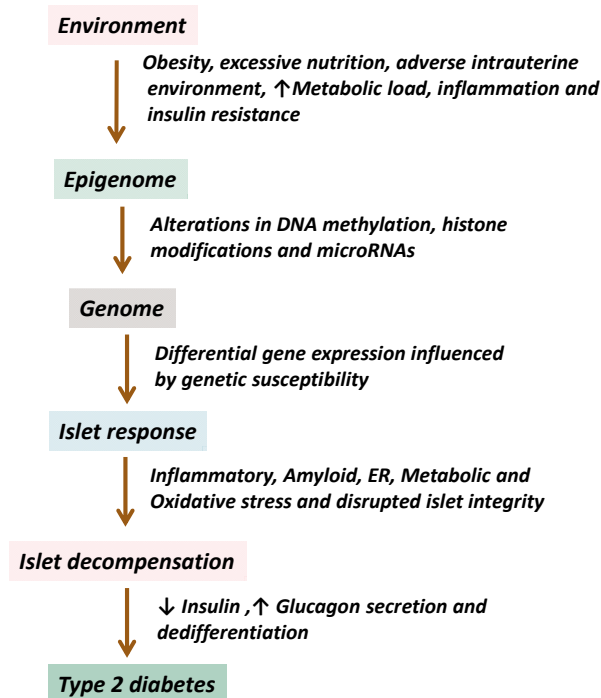


Figure 2. Islet dysfunction in type 2 diabetes.

Environmental, genetic and epigenetic factors contribute to islet dysfunction and T2D. Picture is adapted from [4]

Pancreatic islet architecture

Pancreatic islets are micro-organs that are characterized by their high vasculature structure ensuring efficient endocrine functioning. Each pancreatic islet consists of a number of hormone-secreting endocrine cell types that work together to maintain normoglycemia, including: β -cells (insulin), α -cells (glucagon), δ -cells (somatostatin), PP-cells (pancreatic-polypeptide), and ϵ -cells (ghrelin) [14]. Islet cell types communicate via a number of mechanisms [15]. Differences in islet structure exist

between species. Islets from rodents are characterized by a β -cell core (60–80 percent of islet cells), surrounded by a mantle of other endocrine cells (15–20 percent α -cells, less than 10 percent δ -cells, less than 1 percent PP-cells) (Figure 3). In contrast, endocrine cells in human islets are randomly scattered throughout the islet. In addition, human islets have fewer β -cells and more α -cells than rodent islets (approximately 50 percent β -cells, approximately 40 percent α -cells, 10 percent δ -cells, and a few PP-cells) (Figure 3) [14]. Islet composition and architecture can change in response to physiological and pathological states. Both pregnancy and obesity, which are insulin-resistant states with increased demand for insulin secretion, are associated with an adaptive increase in β -cell mass in humans in response to increased metabolic demands [16-18]. While a decrease in β -cell mass and an increase in α -cell mass have been indicated in some T2D islets, this is still contested [19-22]. T2D islets are, however, characterized by amyloid plaques, and inflammation [23, 24]. The lack of efficient signaling between the different islet cell types in T2D islets, as a result of amyloid plaques, is likely to contribute to impaired hormone secretion [4, 9]. Recently, it has been suggested that some of the hyperglycemia-induced changes in islet architecture are reversible [25].

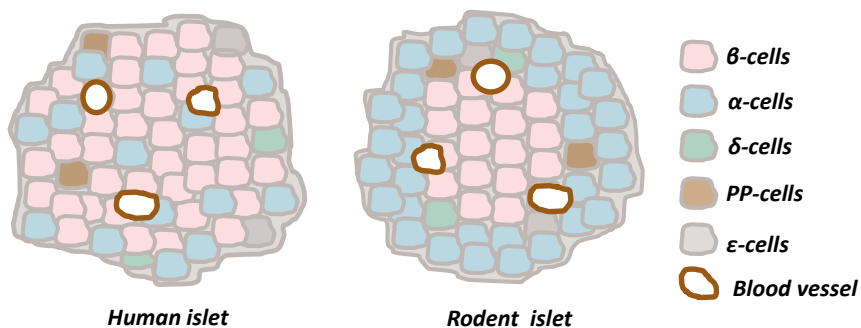


Figure 3. Distribution of islet cell types in human and rodent islets.

Insulin biosynthesis

The insulin (*INS*) gene encodes preproinsulin. Following transcription of the *INS* gene, preproinsulin messenger RNA (mRNA) is transported to the ER where it is translated into the preproinsulin peptide. Preproinsulin is then translocated across the ER to the ER lumen via its signal peptide, which is then cleaved to produce proinsulin (Figure 4). In the ER lumen, proinsulin is folded and disulfide bonds are formed. Proinsulin is then transported to the Golgi apparatus where it is enclosed

into immature secretory granules, and cleaved into insulin and C-peptide, which are both stored and secreted together in the secretory granules [26]. Glucose metabolism is central to the biosynthesis of insulin, and stimulates both insulin gene transcription and insulin mRNA translation, and preserves insulin mRNA stability [26]. T2D islets have reduced proinsulin mRNA levels, with their β -cells containing more uncleaved proinsulin than normal islets [27]. Proinsulin to insulin conversion is impaired in subjects with T2D [28, 29]. Human pancreatic islets that are chronically exposed to high glucose in culture, show reduced insulin mRNA and protein content, together with reduced GSIS [30]. In some forms of neonatal diabetes, insulin folding is impaired, causing ER stress and β -cell apoptosis [31].

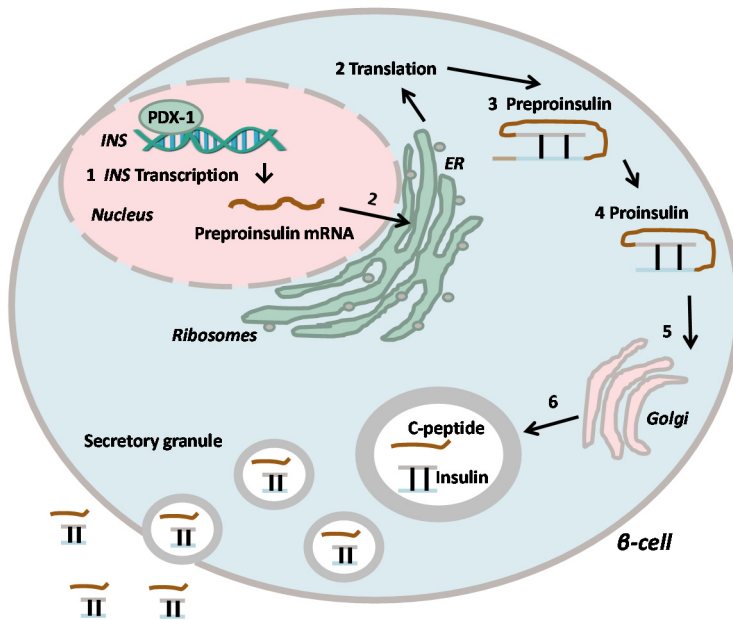


Figure 4. Insulin biosynthesis.

INS gene regulation

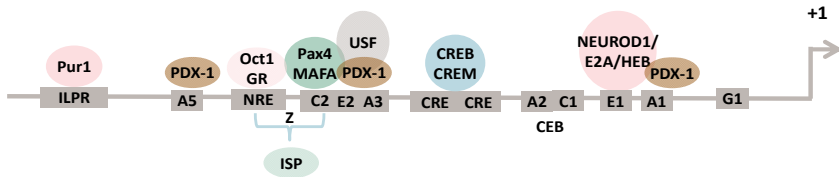
The *INS* gene is mainly expressed in pancreatic β -cells. Transcription of the *INS* gene is regulated by a number of cis-acting regulatory elements located approximately 400 base pairs (bp) upstream from the transcription start site (TSS), to which trans-acting factors bind, including a number of islet-specific transcription factors (TFs) (such as Pdx-1, Mafa and NeuroD1), and ubiquitous TFs (such as E2A) (Figure 5). The TFs interact with each other, and with the deoxyribonucleic acid (DNA), to form complexes. Transcriptional activation is influenced by TF concentration in the

nucleus, and by nutrients (glucose and fatty acids) and hormones (insulin and GLP-1) that can alter the affinity of the TFs for each other and for DNA. In contrast to humans, who have only one copy of the *INS* gene, rodents have two non-allelic copies of the gene, with the rodent *Ins2* being more similar to the human gene. Despite significant differences between species, a number of cis-regulatory elements that are important for glucose regulation are conserved between species, including the pancreatic and duodenal homeobox 1 (PDX-1) binding A3 element [32, 33]. Human pancreatic islets that are chronically exposed to glucose in culture, show reduced PDX-1 binding to the *INS* gene promoter, in parallel with reduced GSIS, insulin mRNA and protein content, and PDX-1 mRNA [30]. Furthermore, mutations in the *INS* gene can cause a rare form of monogenic diabetes, known as Mutant INS-Genotype-Induced Diabetes of Youth (MIDY) [34]. The *INS* gene mutations are localized in the untranslated region of the *INS* gene and the coding sequence of preproinsulin, and disrupt insulin biosynthesis causing proinsulin misfolding and ER stress [35, 36].

PDX-1

PDX-1 is a TF that plays a key role in pancreas development and β -cell function [37]. Although originally expressed in both endocrine and exocrine cells, its expression becomes restricted to pancreatic β -cells during development, where it plays an important role in maintaining β -cell identity and function [38, 39]. In mice, agenesis of the pancreas is caused by targeted inactivation of the *PDX-1* gene, and it is caused by its mutations in humans [40, 41]. Selective disruption of *Pdx-1* in mice leads to diabetes, and reduced insulin and glucose transporter 2 (Glut2) expression [42]. Insulin gene expression requires activation by PDX-1 [43]. PDX-1 mRNA and/or protein expression, and its binding to target sites, is reduced in human pancreatic islets exposed to chronic hyperglycemia, and in rat pancreatic islets exposed to palmitate [30, 44]. PDX-1 expression and binding to its endogenous target gene promoters is reduced in T2D [45]. The *PDX-1* gene is regulated by a number of *cis* and *trans* regulatory elements, some of which are conserved between species (Figure 5). *Cis*-acting elements include a proximal and distal promoter, and an enhancer region. *Trans*-acting elements include PDX-1 itself, and a number of TFs, such as HNF1 α and HNF3 β [37, 46]. Mutations in the *PDX-1* gene itself cause maturity-onset diabetes of the young (MODY4) [47], while mutations or functional impairment of some of the TFs that regulate *PDX-1* cause other forms of MODY (for example, mutations in HNF-1 α cause MODY3) [48]. In addition, a rare frameshift mutation in the *PDX-1* gene has been strongly associated with increased risk of T2D [49].

A Human *INS*, chromosome 11



B Human *PDX-1*, chromosome 13

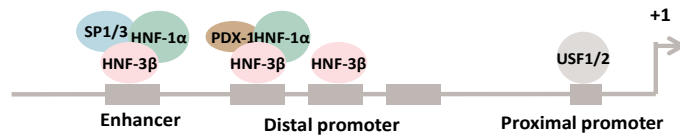


Figure 5. Cis- and trans- regulatory elements of human *INS* and *PDX-1* transcription.

USF= upstream stimulatory factor; HNF= hepatocyte nuclear factor; NEUROD1= neuronal differentiation 1; PAX= paired box; MAFA= v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A; Oct1= octamer transcription factor 1; NRE= negative regulatory element; CRE= cyclic AMP responsive element; PDX-1= pancreatic duodenal homeobox-1; ILPR= insulin-linked polymorphic region; CREM= CRE modulator; CREB= CRE binding protein; Pur1= purine binding transcription factor; ISP= unidentified islet specific protein; Z= minienhancer; CEB= CAAT enhancer binding; GR= glucocorticoid receptor. Figure is adapted from [33, 37]

Insulin secretion

Following an increase in plasma glucose levels, glucose is taken up by the human β -cell via the glucose transporter 1 (Glut1), and phosphorylated by glucokinase (GCK). Glucose-6-phosphate is then metabolized to pyruvate through glycolysis. Pyruvate then enters the mitochondria, and adenosine triphosphate (ATP) is generated by oxidative phosphorylation. The intracellular increase in ATP causes the closure of the ATP-sensitive K_{ATP} channels, leading to calcium influx that triggers exocytosis of insulin granules (Figure 6). This involves translocation and docking of the secretory granules followed by priming, and then fusion with the cell membrane. Insulin secretion is biphasic: first-phase insulin secretion results from the rapid release of insulin granules in close proximity to the Ca^{2+} channels, while second-phase secretion is delayed until the arrival of new granules (Figure 6) [36]. GSIS is defective in islets from T2D donors, and in human pancreatic islets exposed to chronic hyperglycemia in culture [19, 20, 30], with an almost complete lack of first-phase insulin secretion, while second-phase secretion is reduced [36, 50].

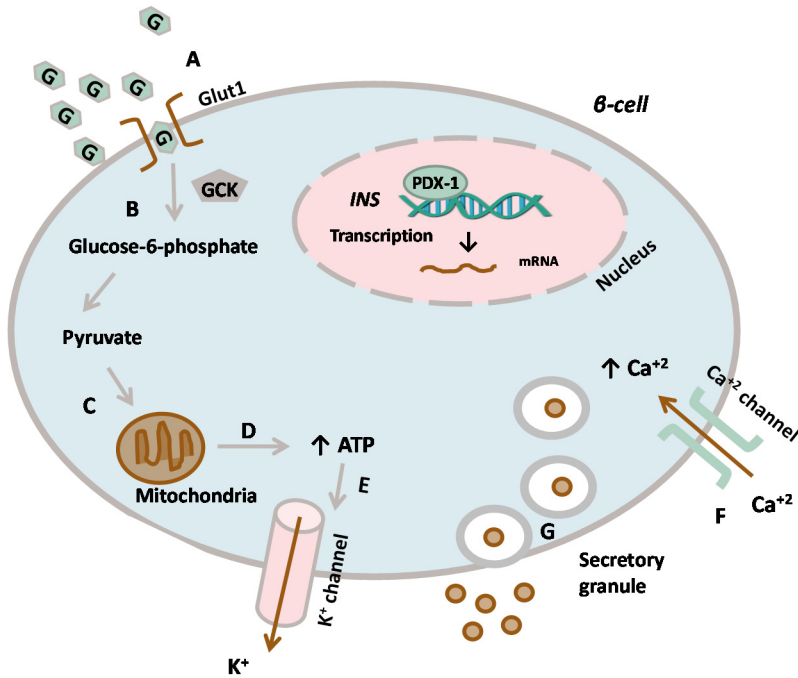


Figure 6. Insulin secretion.

Glucose is uptaken by the human β -cell via the glucose transporter (GLUT1) (A), phosphorylated by glucokinase (GCK) (B) and then metabolized to pyruvate through glycolysis. Pyruvate enters the mitochondria (C). ATP is produced by oxidative phosphorylation (D) resulting in the closure of ATP sensitive potassium (K^+)channels (E). Calcium (Ca^{2+}) influx (F) then triggers insulin granule exocytosis (G).

Genetics of Islet Dysfunction in Type 2 Diabetes

Family-based and twin studies, clearly demonstrate that T2D risk is partly determined by genetics [51, 52]. More than 60 genetic variants have been associated with increased risk of T2D [53-56]. T2D risk variants are associated with different indices of proinsulin processing, insulin secretion and insulin sensitivity, with the majority of variants implicated in β -cell dysfunction [57-59]. In addition, a number of T2D linked variants affect insulin granule docking, and are associated with reduced exocytosis and GSIS [58]. With the exception of a few loci where protein coding can be altered (for example, *PPARG*, *KCNJ11*, *GCKR* and *SLC30A8*), the majority are non-coding, and are more likely to be involved in gene regulation rather than protein coding [54, 60-62]. A number of loci associated with T2D and/or fasting glucose are situated in pancreatic islet enhancers, and may disrupt DNA binding, and islet

enhancer activity [63-65]. For example, individuals carrying the risk allele for the *TCF7L2* intronic variant rs7903146 which has the strongest association with T2D, show increased expression of *TCF7L2*, and impaired insulin secretion [66]. *TCF7L2* encodes a TF that is a master regulator of genes involved in insulin production and processing [67]. The T risk allele is located in open chromatin with greater enhancer activity and accessibility to TFs than the C non-risk allele [68]. Another example is the *MTNR1B* variant, rs10830963, which has been associated with increased T2D risk and fasting glucose, and impaired insulin secretion and action [57, 69, 70]. The increased expression of *MTNR1B* in individuals carrying the risk allele is possibly mediated by the risk allele creating a binding site for the TF NEUROD1, which further increases FOXA2-bound enhancer activity in islets, and *MTNR1B* expression [71]. The *KCNJ11* gene, which harbors one of the T2D associated variants, encodes the pore-forming subunit of the ATP-sensitive potassium channel in β -cells [72]. A variant in the *SLC30A8* gene, which encodes a zinc transporter located in the pancreatic β -cell insulin granules, has been shown to be protective against T2D [73].

Epigenetics of Islet Dysfunction in Type 2 Diabetes

An epigenetic trait has been defined as “a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” [74]. Epigenetic modifications play an essential role in a number of biological processes during development, cell differentiation, imprinting, X-chromosome inactivation, and genomic stability [75]. Although the majority of epigenetic alterations are established during early development, and stably maintained through cell proliferation, some are reversible, and can arise stochastically or in response to the environment [76]. The combination of various epigenetic modifications, such as DNA methylation and histone modifications, is translated into a chromatin state that defines whether a certain part of the genome is active or inactive in a specific cell type, and at a specific time [77].

DNA methylation

In differentiated mammalian cells, DNA methylation occurs almost exclusively at cytosine in cytosine guanine dinucleotides (CpG dinucleotides) [78]. 5-methylcytosine (5meC), also known as the fifth base, is formed by the covalent attachment of a methyl group to the fifth position of the cytosine ring in a CpG dinucleotide (Figure 7) [79]. CpG sites are hot spots for mutations caused by the spontaneous deamination of the 5meC to a thymine. If not repaired, the resulting C-T transition creates the most common point mutation in humans [80, 81]. In contrast to the CpG-poor genome, CpG sites are densely located in CpG islands that

are often associated with TSSs and gene promoters (Figure 8) [75]. The DNA methylation pattern is established during development via the *de novo* DNA methyltransferases, DNMT3a and DNMT3b, and maintained via the DNA methyltransferase, DNMT1, which copies the methylation pattern of the maternal DNA strand to the newly synthesized daughter strand during DNA replication. *De novo* DNA methylation can also occur during postnatal life in response to environmental stimuli. The DNA methylation pattern can be passively removed if it is not copied faithfully, or it can be actively removed via DNA repair and ten-eleven translocation (TET) enzymes (Figure 7) [82].

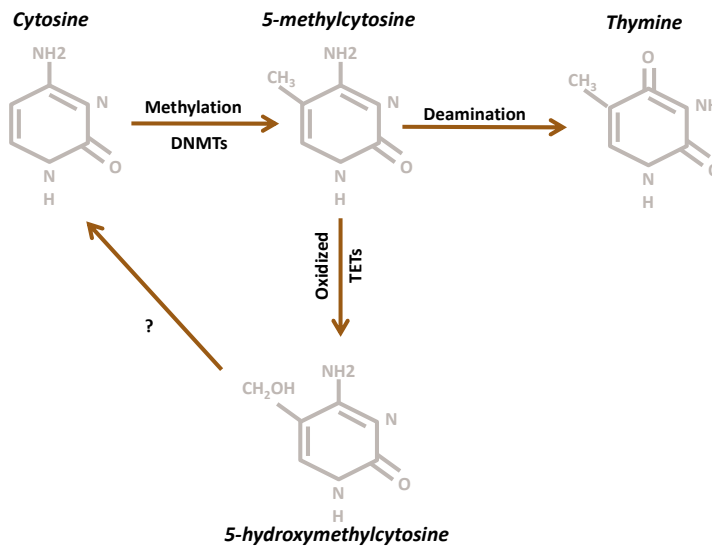


Figure 7. Mechanisms involved in DNA Methylation and demethylation.

DNMTs catalyze the covalent addition of methyl groups to the 5th position of cytosine (forming 5meC). 5meC can be deaminated to a thymine. TET enzymes can catalyze the conversion of 5meC to 5hmC which can then be converted to C.

The non-random distribution of DNA methylation throughout the genome exemplifies its context-dependent diverse roles (Figure 8). For example, with the exception of CpG islands that are generally not methylated, the rest of the genome is mostly methylated. This hypermethylated state plays a key role in maintaining the stability of the genome by repressing repeat sequences, and preventing cryptic initiation of transcription [78]. Promoter DNA methylation is often associated with gene silencing. This repression occurs either directly, by preventing TFs from binding, or indirectly, through the recruitment of methyl CpG-binding proteins that further recruit other chromatin-modifying proteins, such as histone deacetylases (HDACs) [78]. In contrast, gene body methylation is often positively associated with

gene expression, possibly by suppressing the unwanted transcription of intragenic promoters that can interfere with elongation of the main transcript [83]. DNA methylation of non-coding regulatory regions, such as enhancers, can regulate distal promoters by interfering with the binding of TFs and chromatin-modulating proteins, thus affecting physical interactions and DNA looping between the enhancer and distal promoter [84]. Intronic DNA methylation has been associated with alternative splicing events [85].

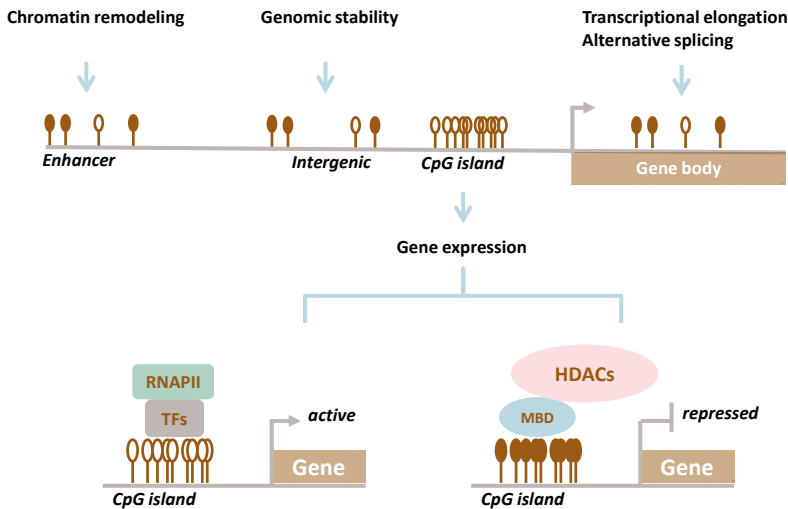


Figure 8. The genome-wide distribution and role of DNA methylation.

RNAPII= RNA polymerase II, TFs= transcription factors, MBD protein = methyl CpG-binding protein, HDACs= histone deacetylases, Filled lollipop= methylated CpGs, Unfilled lollipop= unmethylated CpGs.

Epigenetic regulation of islet function

Pancreatic cell types are derived from the same progenitor cells [86]. Epigenetic modifications play a role in establishing a unique identity for each cell type by allowing the selective expression of certain genes while repressing the expression of other genes. For example, *PDX-1* is initially expressed in all pancreatic cell types, but is later restricted to pancreatic β -cells where it plays a key role in their functional maturation, and the coupling of insulin secretion to glucose levels [87]. In contrast, the expression of *Arx*, a TF that is important for α -cell differentiation and identity, is silenced in β -cells via the methylation of its promoter. Reduced DNA methylation of *Arx* caused by deletion of *Dnmt1* from mouse β -cells leads to ectopic *Arx* expression in β -cells. Consequently, β -cells lose their identity, and convert to glucagon

producing α -cells [88]. For this reason, promoters of genes that are selectively and highly expressed in β -cells, are characterized by active chromatin marks (for example, H3K4me3) around their promoters, by a lack of repressive marks (for example, H3K27me3), and by a low degree of DNA methylation. In contrast, promoters of genes that are selectively repressed in β -cells, are enriched for repressive marks (for example, H3K27me3) [89]. The selective repression of a group of genes, known as β -cell disallowed genes, is of especial importance for the coupling of insulin secretion to glucose levels, and includes *LDHA* and *SLC16A1* [87, 89, 90].

Epigenetic dysregulation of islets in type 2 diabetes

Altered expression caused by epigenetic dysregulation of genes that are important for islet function (for example, insulin biogenesis, secretion, and glucose sensing) can make islet cells dysfunctional. Similarly, epigenetic dysregulation that results in the increased expression of disallowed genes can also contribute to β -cell dysfunction. Indeed, the expression of a number of β -cell disallowed genes is upregulated in β -cells from subjects with T2D [89, 91]. Epigenetic changes that cause a loss of cell identity can make β -cells transdifferentiate into another islet cell type (for example, α -cells), or dedifferentiate into progenitor-like cells, both of which may play a role in diabetic β -cell failure [88, 92].

Several studies have shown that some of these epigenetic changes can be environmentally induced in pancreatic islets, and contribute to impaired islet function in T2D. For example, offspring of women exposed to the Dutch Hunger Winter (famine) during World War II were born with low birth weights, and had impaired glucose tolerance later in life and less DNA methylation at the imprinted *IGF2* gene than their unexposed siblings [93-95]. Likewise, rats exposed to an adverse intrauterine environment showed epigenetic dysregulation, and altered expression of genes important for β -cell development and differentiation. Moreover, some of the changes persisted into adulthood, and contributed to impaired insulin secretion and diabetes [96, 97]. Interestingly, some of the epigenetic changes could be reversed permanently by treating the newborns with Exendin-4 (a GLP-1 analogue), which preserved *Pdx-1* transcription and prevented its promoter from being methylated [98].

As another example, children born to obese parents often have altered DNA methylation in a number of imprinted genes [99]. Female rats whose fathers had been exposed to a high fat diet had β -cell dysfunction and impaired glucose tolerance, and altered DNA methylation and expression of a gene important for lipid metabolism [100]. In addition, altered DNA methylation, and expression of a number of imprinted genes in human pancreatic islets, including *GRB10*, *KCNQ1*, *PEG3* and *DLK1-MEG3*, has been associated with T2D [101-104]. Exposure of human pancreatic islets to palmitate for 48 hours caused altered DNA methylation, and

expression of a number of genes, together with impaired insulin secretion [105]. Concordantly, exposing clonal rat β -cells to palmitate for 48 hours caused changes in histone modifications, activity of histone modifying enzymes, mRNA levels, and metabolites, as well as decreased GSIS [106].

Translational implications of the islet epigenome in type 2 diabetes

Epigenetic profiling of human pancreatic islets during health and disease can be of great value in our quest to treat or prevent T2D [8]. Importantly, this will help us to identify the types and locations of epigenetic modifications that are important for islet identity and function. Furthermore, it will be useful to understand which environmental factors induce these epigenetic changes and when, and if they can be reversed. The following treatment strategies will benefit from such knowledge:

Restore or enhance islet function

At the time of diagnosis, β -cell dysfunction is already present, and it progressively worsens as T2D progresses. Central to the treatment of diabetes is to preserve β -cell function from further deterioration, and even restore it, if possible [5]. In support of this, it has been shown that some of the changes in islet structure and function caused by hyperglycemia can be reversed [25]. Some T2D drugs, such as GLP-1, gastric inhibitory polypeptide (GIP), and Exendin-4, have an effect on epigenetic modifications [98, 107]. HDAC inhibitors have been suggested as a novel treatment for T2D, and have been shown to enhance insulin secretion and promote β -cell development, proliferation, differentiation and function [108, 109]. Silencing of β -cell disallowed genes via epigenetic editing may also provide a novel approach to improve GSIS [89, 110].

Replacement

Current attempts to generate functional β -cells from stem cells, to be used in both replacement therapy in diabetes and for drug testing, have succeeded in producing β -like cells, but they are not as good as β -cells [7]. It will be interesting to see if modifying stem cells epigenetically in a manner similar to β -cells may improve their function.

Regeneration

Even more challenging are the current attempts to make drugs that can regenerate β -cells in patients without the need for transplantation [8]. In support of this notion, it

has been shown in mice that ectopic expression of *PAX4* [111], and extreme β -cell loss, can induce α -cells to convert to β -cells [112]. Furthermore, adult duct lining cells can also be reprogrammed to β -like cells [113]. It has been suggested that this interconversion may be facilitated by the epigenomic plasticity of α -cells that harbor bivalent (both activating and repressing) epigenetic marks in β -cell specific genes [114].

Biomarkers

Epigenetic changes that occur at an early stage in development are likely to affect multiple tissues, including accessible tissues such as blood. It has been shown that DNA methylation patterns in accessible tissues may reflect changes in target tissues that are difficult to obtain [115-117]. At present, researchers are trying to find DNA methylation biomarkers in blood that can predict future T2D [118, 119]. Interestingly, an assay has been developed to monitor β -cell death in Type 1 diabetes (T1D) at an early stage by detecting β -cell derived unmethylated *INS* gene DNA in the blood [120-122].

Aims of this thesis

The overall aim of this thesis was to investigate if T2D is associated with altered DNA methylation in human pancreatic islets. In addition, we wanted to study possible interactions between epigenetic, genetic and environmental factors, and to examine whether DNA methylation in blood DNA could be used to predict future T2D.

The specific aims were as follows:

- | | |
|------------------|--|
| Studies I and II | To investigate whether epigenetic alterations of <i>INS</i> and <i>PDX-1</i> in human pancreatic islets are associated with impaired insulin secretion in T2D. |
| Study III | To describe the human pancreatic islet genome-wide methylome, and its role in impaired islet function and T2D. |
| Study IV | To examine whether any of 40 SNPs associated with T2D, introduce or remove a CpG site, and if these CpG-SNPs are associated with differential DNA methylation in human pancreatic islets. |
| Study V | To examine whether five recently reported T2D-associated DNA methylation loci in blood DNA (<i>ABCG1</i> , <i>PHOSPHO1</i> , <i>SOCS3</i> , <i>SREBF1</i> and <i>TXNIP</i>) predict future T2D in subjects from the Botnia prospective study; and, by comparing diabetic versus non-diabetic subjects, to test if DNA methylation of these CpG sites is altered in human pancreatic islets, liver, adipose tissue and skeletal muscle. |

Study participants

All studies were approved by the regional ethical committees, and conducted in accordance with the *Declaration of Helsinki*. Informed consent was obtained from all study participants, and from the pancreatic islet donors (or their relatives) to donate organs for medical research upon admission to the intensive care unit.

Human Pancreatic Islets

Human pancreatic islets from up to 15 T2D donors and 84 non-diabetic donors were included in this research (Table 1). Human pancreatic islets and clinical characteristics of donors were obtained from the Nordic Network for Islets Transplantation [123]. Following the procurement of the pancreas from brain dead/cadaveric donors, enzymes were used to further dissect the islets from exocrine material. Islets, which have a lower density than exocrine tissue, were then further separated by gradient centrifugation. Dithizone (DTZ) staining was used to determine the purity of islets. Additionally, the quality of the islets was assessed by measuring: dynamic glucose perfusion, insulin/DNA content, ADP/ATP ratio and cytokines [124]. Upon arrival at the Human Tissue Laboratory (HTL) at Lund University Diabetes Center (LUDC), insulin and glucagon secretion were measured during static glucose stimulation. Additionally, DNA and RNA were extracted, and genetic and transcriptomic profiling were performed [125]. Human pancreatic islet donors were considered to have T2D only if they had been diagnosed with T2D prior to their death, regardless of their glycosylated hemoglobin (HbA1c) levels.

Table 1.
Clinical characteristics of human pancreatic islet donors.

Characteristics	Non-diabetic	T2D
n (male/female)	84 (32/52)	15 (10/5)
Age (years)	57.1 ± 10.3	59.5 ± 10.7
BMI (kg/m ²)	25.9 ± 3.6	28.3 ± 4.7
Hb A1c (%)	5.6 ± 0.4	6.9 ± 1.0

Data are presented as mean ± SD.

Purified Human β - and α -cells

Human pancreatic β - and α -cells were purified from three donors (Table 2). The islets were provided through islet isolation centers at the University of Pisa in Italy and the Geneva University Hospital in Switzerland. β -cells were purified using the Newport green labeling method [126].

Table 2.
Clinical characteristics of donors of purified β - and α -cells.

Characteristics	Donors of sorted β - and α -cells
n (male/female)	3 (2/1)
Age (years)	61.0 \pm 9.2
BMI (kg/m ²)	24.0 \pm 2.4

Data are presented as mean \pm SD.

Botnia Prospective Study

The Botnia prospective study is a family-based study that was initiated in 1990 to identify early metabolic defects in families with T2D. The study includes 2,770 participants from the Botnia region of Finland who were non-diabetic at baseline. Oral glucose tolerance tests (OGTTs) were regularly performed to detect progression to T2D. DNA was extracted from whole blood taken at baseline [127]. In Study V, we used DNA from 129 participants who had developed T2D (converters) after a mean follow up time of 8.1 \pm 3.7 years, and we also selected 129 controls whom were matched for age and gender at baseline and had not developed T2D over the same follow-up period. Their baseline characteristics are shown in Table 3.

Monozygotic Twins

Monozygotic (MZ) twin pairs, discordant for T2D, were obtained from the Scandinavian twin registry [128]. MZ twins are a good model for studying environmental contributions to T2D while eliminating confounding factors, such as genotype, age and gender [129]. In Study V, we used genome-wide DNA methylation and expression data from adipose tissue, skeletal muscle and blood cells from up to 19 MZ twin pairs discordant for T2D (Table 3). Some of this data has previously been published [130-132].

Kuopio Obesity Surgery Study

The Kuopio obesity surgery study (KOBS) includes subjects who had undergone bariatric surgery at the Kuopio University Hospital in Finland. The aim of the study was to investigate metabolic consequences of bariatric surgery and non-alcoholic steatohepatitis (NASH) [133]. In Study V, we used published human liver DNA methylation data from 95 participants from KOBS (35 with T2D and 60 non-diabetic subjects) (Table 3). Expression data from a subset of the participants (19 T2D and 23 non-diabetic subjects) was also included in the study [134].

Table 3.
Clinical characteristics of participants in case-control cohorts.

	Botnia (n=258)		Twin pairs (n=19)		KOBS (n=95)	
	Control	T2D	Control	T2D	Control	T2D
male/female	62/67	65/64	11/8		8/42	17/18
Age (years)	51.4 ± 9.1	52.8 ± 12.3	66.5 ± 8.0		48.7 ± 7.8	50.5 ± 7.2
BMI (kg/m ²)	27.6 ± 3.0	28.8 ± 4.3	29.1 ± 6.1	30.9 ± 6.4	43.6 ± 5.5	42.1 ± 6.0
FPG (mmol/L)	5.1 ± 0.4	5.3 ± 0.6	6.2 ± 0.6	9.7 ± 3.0	5.8 ± 0.8	7.7 ± 2.9

Data are presented as mean ± SD. FPG = Fasting plasma glucose.

Methodology

Phenotype Characterization

Participants in the various cohorts included in this thesis have been phenotypically characterized by a number of metabolic and anthropometric parameters, including the following:

Body mass index (BMI): BMI is a simple index used to classify obesity, and is calculated by dividing weight (kg) by the square of the height (m²). According to definitions by the World Health Organization (WHO) [135], an individual with a BMI ≥ 25 is considered to be overweight, while a BMI ≥ 30 is an indication of obesity.

Fasting plasma glucose (FPG): In this test, plasma glucose levels are measured in an individual following overnight fasting. According to the WHO [136], an individual with an FPG ≥ 7.0 mmol/L is considered to be diabetic.

2 hour plasma glucose (2hrPG) after an (OGTT): Following an overnight fast, an individual is given 75g of glucose orally, and then plasma glucose is measured over the next two hours, often after 30, 60 and 120 minutes. According to the WHO [136], an individual with a 2hrPG ≥ 11.1 following an OGTT, is considered to be diabetic.

HbA1c : Glycated hemoglobin (HbA1c) is a measure of the average plasma glucose over the previous eight to 12 weeks. An individual with HbA1c ≥ 6.5 is considered to be diabetic. However, according to the WHO [137], having an HbA1c < 6.5 does not exclude diabetes. HbA1c can be measured by a number of techniques, and can be presented in different units [138]. In this thesis, the mono S method, which is a method based on high-performance liquid chromatography (HPLC), was used to measure HbA1c.

Characterization of Human Pancreatic Islets

Islet Purity

DTZ staining

DTZ staining is a routine method used to assess the purity of islets relative to non-islet material. In this method, DTZ binds to the zinc-insulin complexes in β -cell granules, causing the islets to stain red in contrast to the yellow-brown/white exocrine material (Figure 9). A light microscope is then used to count the number of islet equivalents (IEs), and to determine the purity of the islets [139, 140]. The purity of human pancreatic islets used in this thesis was determined by DTZ staining performed at the transplantation center.

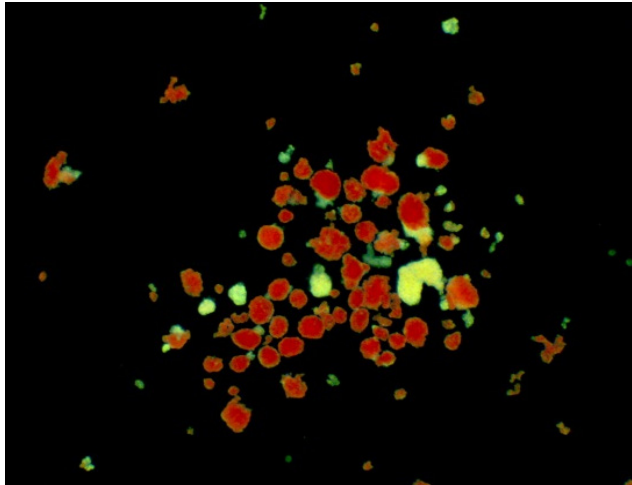


Figure 9. DTZ staining of human pancreatic islets. Islets are stained in red, while non-islet material appears yellow-brown/white. Courtesy of Dr. Magnus Stähle.

Expression of cell-specific genes

Islet purity was also assessed by quantifying mRNA expression, and determining the ratio of endocrine-specific genes (somatostatin and glucagon) to exocrine-specific genes (pancreatic lipase, amylase α 2A, and chymotrypsin).

Electron microscopy (EM)

In Study III, we used EM to determine the cellular composition of human pancreatic islets. Islets were handpicked, fixed, dehydrated, embedded in agar, and then cut into sections. The sections were then put onto grids, and prepared for EM. Micrographs were analyzed and islet cell types were distinguished based on their granularity [139, 141]. β -cell granules have a dense core surrounded by a white halo, while α -cells have small dense granules (Figure 10). The ratio of β -cells in each islet was calculated by dividing the number of β -cells by the total number of β - and α cells.

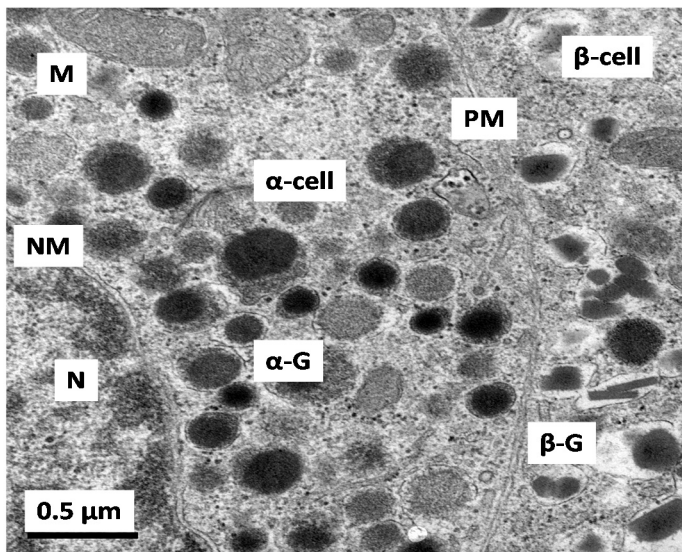


Figure 10. Electron micrograph of a human pancreatic islet.

β -cell granules have a dense core surrounded by a white halo, while α -cells have small dense granules. N=nucleus, NM=nuclear membrane, PM=plasma membrane, M=mitochondria, G=granules. Courtesy of Professor Lena Eliasson.

Hormonal secretion

Dynamic glucose stimulation (perfusion)

In this assay, a perfusion apparatus is used, and islets are continuously perfused for a certain amount of time with a buffer that contains different concentrations of glucose: low (1.67 mM) or high (16.7 mM). The perfusates are collected every 6 minutes, and insulin is quantified using an enzyme-linked immunosorbent assay (ELISA). The concentration of insulin is determined by using the measured absorbance, and by

relating it to known concentrations from a standard curve. The stimulation index (SI) is calculated as the average concentration of insulin secreted at high glucose divided by the average insulin concentration secreted at low glucose (Figure 11) [140, 142]. In addition, intracellular insulin content was measured in homogenized human islets using an ELISA, and normalized to total DNA measured by Pico green, which is a dye that binds to double stranded DNA.[142].

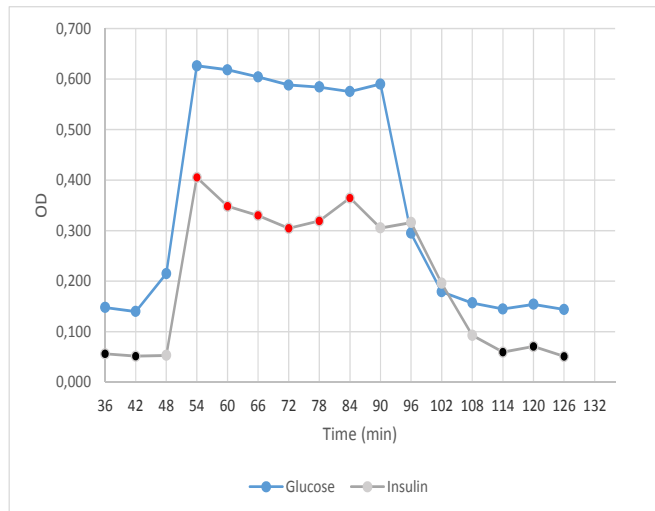


Figure 11. Measuring insulin secretion in human pancreatic islets using dynamic glucose stimulation. The SI is calculated as the average concentration of insulin secreted at high glucose (red points) divided by the average insulin concentration secreted at low glucose (black points). Courtesy of Dr. Magnus Ståhle.

Static glucose stimulation

In this method, islets were incubated with low (2.8 mM) or high (16.7 mM) glucose in static incubations for one hour. The amount of insulin released in the medium was then measured with a radioimmunoassay (RIA), and normalized to the number of islets [140, 143]. Glucagon secretion was also measured in static incubations by incubating human pancreatic islets with 8.3 mM or 16.7 mM glucose for one hour. The amount of secreted glucagon in the medium was then measured using a RIA, and normalized to the number of islets.

Fluorescence-activated Cell Sorting of Human β - and α -cells

Human pancreatic islet β - and α -cells were sorted using the Newport green labeling method. The method takes advantage of the high zinc content that is found in β -cells and is used in the packaging of insulin. Newport green is a non-toxic zinc sensitive fluorescent probe [144, 145]. After dissociating islets with trypsin, the cells were stained with Newport green (stains β -cells), anti-7-AAD (stains dead cells) and anti-CA19-9 (stains ductal cells) and sorted by fluorescence-activated cell sorting (FACS). Cells that were positive for Newport green (FL1), negative for 7-AAD and CA19-9 and were granular (based on their SSC) were considered to be β -cells. Cells that were negative for Newport green and had fewer granules were considered to be α -cells (Figure 12). Immunohistochemistry was used to determine the purity of the sorted cells by co-staining them with anti-insulin and anti-glucagon antibodies. The purity of the β -cell fraction was 89 percent, and the purity of the α -cell fraction was 75 percent [126].

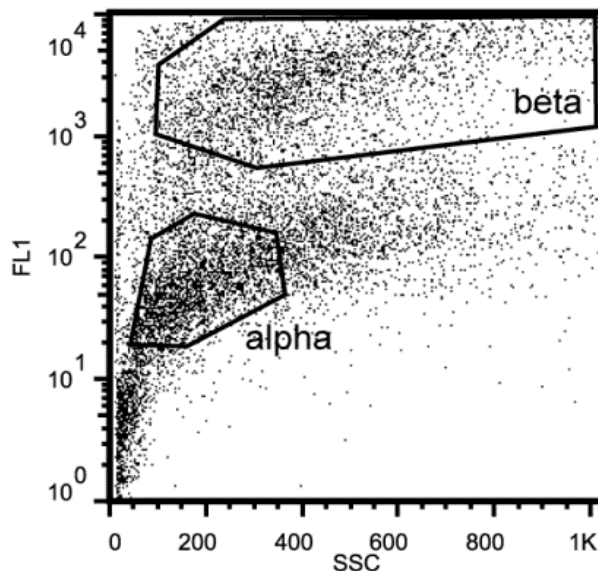


Figure 12. FACS plot of sorted human pancreatic β - and α -cells. Newport green staining (FL1), granularity (SSC). Courtesy of Dr. Clare Kirkpatrick.

DNA Methylation

Bisulfite conversion

During DNA replication of cells, the DNA methylation pattern is faithfully copied to the newly-synthesized daughter strand via the DNA methyltransferase DNMT1 [146]. However, this is not the case *in vitro*, and a few rounds of DNA amplification are sufficient to remove the majority of methyl groups. A solution to this problem was the bisulfite conversion method that was first introduced by Frommer et al. in 1992 [147]. In this method, single-stranded DNA is treated with sodium bisulfite under acidic conditions. This causes deamination of cytosine to uracil, while 5-methylcytosine (5meC) is not converted. Bisulfite conversion of DNA yields DNA strands that are not complementary to each other, and amplification of the DNA strands requires strand-specific primers. During polymerase chain reaction (PCR) amplification, uracil (formerly cytosine) and thymine residues are amplified as thymine, while 5meC is amplified as cytosine [147]. The methylation-dependent changes in the DNA sequence can then be detected, and measured by a number of methods based on differences in mass, sequencing, or probe hybridization. The following section describes three methods that were used to analyze DNA methylation in this research.

EpiTYPER

EpiTYPER is a mass spectrometry-based method that can analyze DNA methylation of a specific region with a high throughput, and in a quantitative manner [148]. In this method, bisulfite-converted DNA is amplified using custom designed CpG-free primers for the region of interest, and with one primer tagged with a T7 promoter sequence (Figure 13). After the removal of unincorporated nucleotides using shrimp alkaline phosphatase (SAP), the amplified DNA strand in which the T7 promoter sequence is incorporated is then reverse transcribed *in vitro* into a single-stranded RNA molecule. Ribonuclease (RNase) A, which is an endoribonuclease, is then used to cleave the RNA molecule in a base-specific manner. After the removal of unwanted salts with a resin, the samples are loaded onto a SpectroCHIP II array. Subsequently, the cleavage products are analyzed by Matrix-Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS), and the energy produced by the laser is transferred to the RNA fragments, which become ionized. The time it takes the ionized RNA fragments to reach the detector depends on their mass, with heavier fragments needing more time [149].

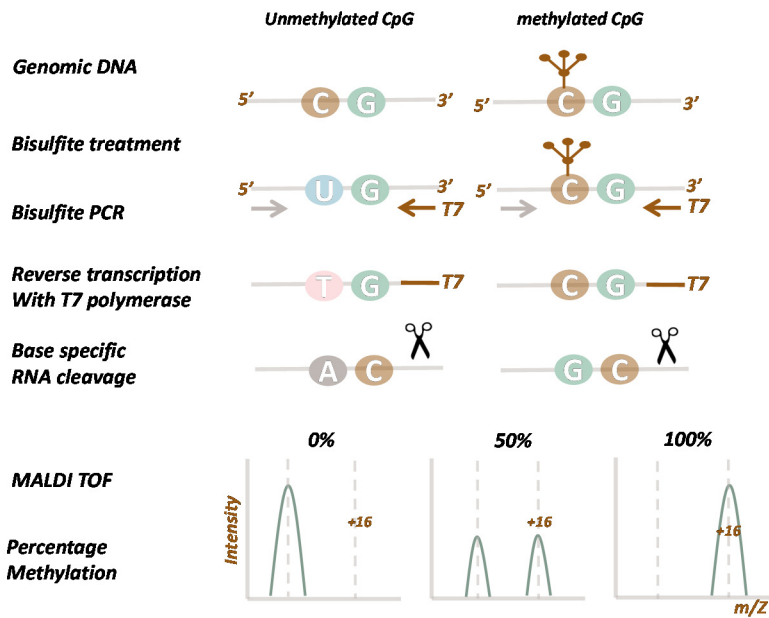


Figure 13. Analyzing DNA methylation with EpiTYPER. Picture is adapted from [149].

The 16 dalton difference in mass between G (methylated C) and A (unmethylated C) is used to distinguish methylated from unmethylated fragments of the same cleavage product (Figure 13). The area under a peak is used to determine the number of fragments with a specific molecular weight. The degree of DNA methylation at a specific CpG site, or a number of CpG sites, is the surface area under the peak corresponding to the methylated fragment divided by the surface area of the peaks corresponding to both methylated and unmethylated fragments [149]. In this thesis, EpiTYPER was used to analyze DNA methylation of the *INS* promoter in Study I, and the *PDX-1* promoter and enhancer regions in Study II.

Pyrosequencing

Pyrosequencing is a real time DNA sequencing method that is based on sequencing by synthesis [150]. The name is derived from the pyrophosphate that is released when nucleotides are incorporated. The method uses strand-specific primers that are designed for the region of interest, and one of the primers is biotinylated on its 5' end. Following PCR amplification, the DNA is denatured. The biotinylated strand, which is immobilized onto streptavidin beads, will then anneal to the sequencing

primer. A mixture of enzymes and substrates is added, and then nucleotides are added sequentially based on the target region sequence. Upon the incorporation of a nucleotide complementary to the DNA strand, pyrophosphate is released, and is enzymatically converted to a luciferase light that is detected. The cytosine in a CpG site can incorporate a C (methylated C) or T (unmethylated C). The ratio of the signal obtained from both C and T represents the percentage of methylation at that CpG site (Figure 14) [151]. In this thesis, we used pyrosequencing in Studies I and II to analyze DNA methylation of CpG sites that were difficult to analyze with EpiTYPER, in Study IV to analyze DNA methylation at CpG-SNP sites, and in Studies III and V to replicate and validate genome-wide DNA methylation data.

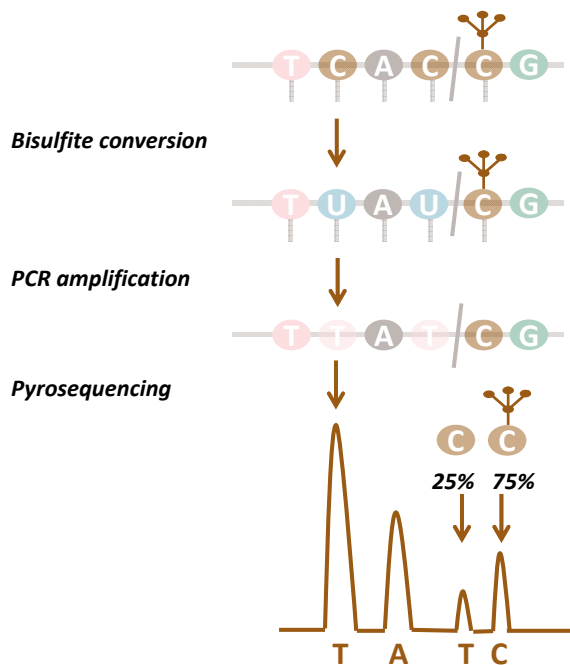


Figure 14. Using pyrosequencing to analyze DNA methylation.

The amount of luciferase light released during pyrosequencing is proportional to the number of nucleotides added. The % of DNA methylation at a CpG site is the ratio of the signal obtained from C (methylated C) divided by the signal obtained from both C and T (unmethylated C).

Infinium HumanMethylation450 BeadChip

The Illumina Infinium HumanMethylation450 BeadChip (450k array) is a powerful tool for genome-wide association studies. The array contains probes to analyze more than 450,000 CpG sites, covering 99 percent of RefSeq genes, and is designed to enable comprehensive coverage of all gene regions. Probes are annotated to different genomic regions or CpG island regions (Figure 15) [152]. The 450k array utilizes two different chemistries. The Infinium I probe chemistry has two beads per CpG site: one for the methylated CpG site, and another for the unmethylated CpG site. Upon hybridization to the bisulfite-converted DNA, a labeled nucleotide is added that is complementary to the base following the CpG dinucleotide. The signal detected from both probes is read in the same color channel (Figure 16A). In contrast, the Infinium II chemistry uses one bead for each CpG site, and the methylation state at the CpG site is determined by single base extension with fluorescently-labeled nucleotides. Addition of an A (unmethylated) will give a different color than addition of a G (methylated) (Figure 16B) [152].

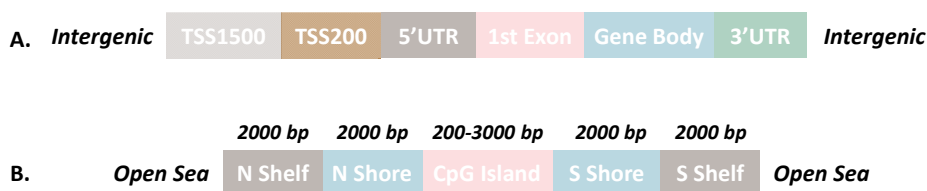
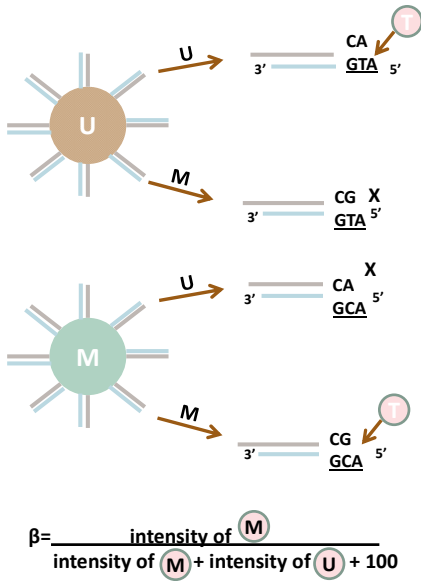


Figure 15. Annotation of 450k array probes.

Probes on the 450k array are annotated to different genomic regions based on their relation to the closest gene (A), or to a CpG island (B) [152]. TSS1500: 200-1500bp upstream from the transcription start site (TSS), TSS200: 200bp upstream from the TSS, UTR: untranslated region, CpG island: 200bp (or more) stretch of DNA with a GC content >50% and an observed CpG/expected CpG >0.6, Shore: up to 2000bp flanking region of CpG island, Shelf: regions flanking island shores (2000-4000bp) from CpG island, N: north, S: south.

We then used the obtained fluorescence intensities to calculate the methylation at each CpG site, also known as the β -value: β -value = (intensity of methylated allele)/(intensity of both methylated and unmethylated allele +100) (Figure 16). Following the filtration of unreliable probes, the β -values were converted to M-values, which are more suitable for statistical analysis because of their lower heteroscedasticity (variance across the methylation range is approximately constant). We used data from negative control probes to correct for background fluorescence, and quantile normalization for between-array normalization (Figure 17) [153, 154].

A. Type I (2 probes per CpG, one color readout)



B. Type II (1 probe per CpG, two color readout)

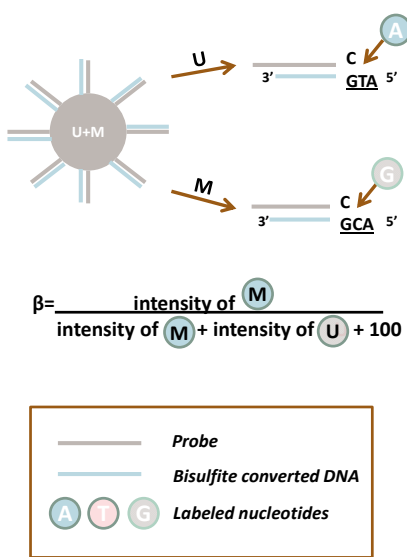


Figure 16. The chemistry of type 1 and II probes used in the 450k array.

A. Type I unmethylated probes (U) will only give a signal if they hybridize to bisulfite converted DNA that was not methylated at the CpG site, while the methylated probes (M) only give a signal if they hybridize to a DNA fragment that was methylated at the CpG site. Both probes give the same signal. B. In contrast, Type II probes will hybridize to both methylated (M) and unmethylated (U) bisulfite converted DNA, but will give a different signal based on the methylation status. Picture is adapted from [155].

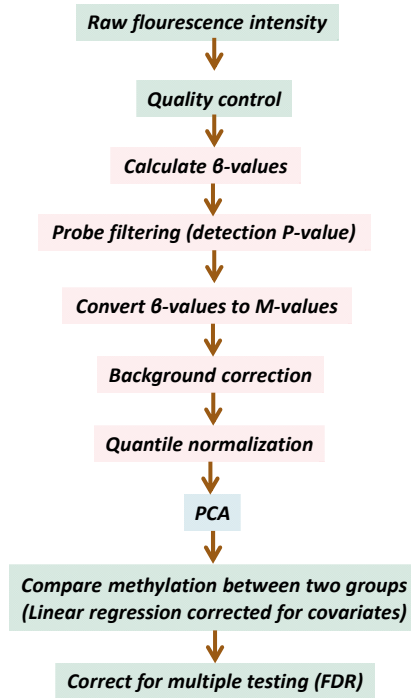


Figure 17. Overview of the pipeline that was used to analyze DNA methylation data generated by the 450k array.

PCA = principle component analysis. FDR = false discovery rate.

Gene Expression

In this thesis, two methods were used to measure gene expression. We used TaqMan real time PCR expression assays, which can be used to study the expression of a single gene, to quantify the expression of target genes in human pancreatic islets, and to verify the silencing or overexpression of target genes in cell lines. We used gene expression microarrays to analyze expression genome-wide in human pancreatic islets, adipose tissue, skeletal muscle and liver.

Reverse transcription (RT) is the first step prior to RNA quantification. During RT, the RNA template is converted into complementary DNA (cDNA) via an RNA-dependent DNA polymerase, also known as reverse transcriptase [156]. For this thesis, we used oligo dT and random primers to prime the RT reaction.

TaqMan real time PCR

The TaqMan real time PCR expression assay (Applied Biosystems) is a widely used method for nucleic acid quantification. This method takes advantage of the 5' exonuclease activity of Taq polymerase. The assay consists of a set of primers that are designed for a specific target, and a probe with a fluorescent dye on the 5' end and a quencher on the 3' end. During the extension phase of PCR, the probe will anneal to a perfectly-matching sequence in the target DNA. Then the 5' exonuclease activity of Taq DNA polymerase cleaves the probe, causing the dissociation of the quencher from the reporter, only if a target sequence complementary to the probe is amplified. As a result, a detectable fluorescent light is released during every cycle. The amount of fluorescent dye is proportional to the amount of cDNA in the starting material. The more cDNA in the starting material, the fewer the number of cycles needed to reach a point in which a fluorescent signal is detected, also known as the cycle threshold (Ct value). To account for possible technical errors (for example, variation in the amount of starting material), the data is normalized to reference genes that are expressed at constant levels and not affected by the experiment [156].

Gene expression microarrays

Gene expression microarrays are powerful tools that can give a comprehensive picture of all the transcriptional activity in a biological sample [157]. In this thesis, we used two different microarrays to analyze gene expression genome-wide. To account for technical variation without affecting biological variation, we used the Robust Multi-Array average (RMA) normalization method [158]. We used ComBat to account for batch effects [159].

GeneChip Human Gene 1.0 ST array

The Affymetrix microarray includes 764,885 distinct probes that cover 28,869 well-annotated genes. In this array, 25-mer probes are printed base by base on the array. For each gene, 27 probes are designed that cover the full length of the gene [160]. Expression values obtained from all probes of a gene are summarized into one value. Alternatively, it is possible to obtain isoform-specific expression data [161].

HumanHT-12 Expression BeadChip

The HumanHT Expression BeadChip (Illumina) includes more than 47,000 probes that cover 26,228 coding transcripts and some non-coding transcripts. In this array, 50-mer probes are immobilized to magnetic beads distributed across the array, with

up to 30 beads per probe. The majority of genes are represented by one probe, while some are represented by two probes for different isoforms [162].

Genotyping

In Study IV, we used two methods for genotyping. We used the Human OmniExpress BeadChip to genotype 12 SNPs associated with T2D in human pancreatic islet donors, and we used the TaqMan allelic discrimination assays to genotype four SNPs that were not included on the Human OmniExpress BeadChip.

Human OmniExpress BeadChip

The Human OmniExpress BeadChip (Illumina) is a powerful tool for studying common variants. The BeadChip contains more than 700,000 tag SNPs that were selected from three phases of the HapMap project, allowing for the greatest coverage of common variants (minor allele frequency (MAF) greater than five percent). The principle behind the tag SNPs is that, when SNPs are in linkage disequilibrium (LD) and correlate with each other, genotyping one of them can provide information about the other without the need to genotype it. Thus using tag SNPs allows for maximal genome-wide coverage without the need to have a probe for every single SNP on the array. Beadchips are covered with hundreds of thousands of copies of 50-mer probes that are immobilized to beads. The hybridization of the DNA fragment to the probe is followed by an enzymatic single-base extension, and the incorporation of a labeled nucleotide. The assay uses two color readouts, one for each allele [163].

TaqMan allelic discrimination

The TaqMan allelic discrimination assay (Applied Biosystems) is a simple method that is used to genotype a single SNP. It uses the same chemistry as the TaqMan expression assay, which includes: hybridization of a probe, cleavage, and release of a fluorescent dye. However, the main difference is that it uses two probes instead of one. Each probe is specific to an allele, and has a distinct fluorescent dye. Individuals that are homozygous for an allele will give a strong signal from that probe, while heterozygous individuals will give a signal from both probes [164].

Functional Experiments

Cell line models

In this thesis, the following two cell line models were used to study the effects of hyperglycemia on DNA methylation of *INS* and *PDX-1* in Studies I and II, and to study the effects of altered DNA methylation/expression of target genes on β - and α -cell function in Studies II and III:

Rat INS-1 832/13 clonal β -cells

The INS-1 β -cell line was established from cells isolated from an X-ray induced rat insulinoma. Despite their ability to synthesize proinsulin, the insulinoma cells were not very responsive to glucose [165]. The 832/13 cell line was clonally selected from INS-1 cells that were stably transfected with a plasmid containing the human proinsulin gene, and was strongly responsive to glucose as well as other potentiators [166]. Therefore, the INS-1 832/13 clonal β -cells provide a good model for studying various aspects of β -cell function.

Mouse α -TC1-6

The α -TC1-6 cells were derived from the α -tumor cell 1 (α -TC1) line which was derived from a glucagonoma arising in transgenic mice expressing the SV40 large T antigen oncogene under the control of the rat preproglucagon promoter [167]. Unlike the α -TC1 parental cell line, which produced both glucagon and insulin, the α -TC1 clone 6 is more differentiated, and produces only glucagon [168]. The α -TC1 clone 6 cells provide a useful model for studying glucagon biosynthesis.

Insulin secretion

In Study III, we wanted to test if β -cells overexpressing *Cdkn1a*, *Pde7b*, and *Sept9* secreted less insulin than control cells. To do so, INS-1 832/13 β -cells were transfected with pcDNA3.1 expression vectors containing cDNA for any one of *Cdkn1a*, *Pde7b* or *Sept9*. After 48 hours of transfection, GSIS was measured.

Transfection

Transfection is a method that is used to insert genetic information into cells of an organism. Transfection can be achieved by physical, chemical or biological methods.

Lipofection is a transfection method that is often used in cell lines, and is considered to be a simple and fast method with high reproducibility [169]. In this method, positively charged cationic lipids interact with the negatively charged phosphate backbone of nucleic acids; this compact structure, together with other lipids, interacts with the cell membrane, and is then uptaken by the cell. In this thesis, we used three different lipofection-based transfection reagents in Studies II and III.

Glucose-stimulated insulin secretion (GSIS) assay

Prior to measuring insulin secretion, cells were incubated in a HEPES balanced salt solution (HBSS), supplemented with 2.8 mM of glucose for two hours at 37°C. The cells were then stimulated to secrete insulin by incubating them with HBSS, supplemented with the desired secretagogue: 2.8 mM glucose (for basal secretion), 16.7 mM glucose (for GSIS), and 35 mM K⁺ (for first phase insulin secretion) for one hour at 37°C. We collected the supernatant, and then lysed the cells with water, and scraped and sonicated them to stimulate them to release their content. We then used centrifugation to remove insoluble material. We used acid ethanol to extract insulin. We then diluted the samples, and used a RIA to measure insulin secretion and content. Insulin secretion was normalized to insulin content.

Acid ethanol extraction

Acid ethanol extraction is a method that is used to dissociate proteins from each other. The method was originally used to remove insulin-like growth factor binding proteins (IGFBP) from IGF. The presence of the IGFBP interfered with the RIAs used to measure IGF by partially blocking the epitopes that are targeted by the antibodies, and thus leading to an underestimation of the real amount of IGF. The acidic conditions used in this method cause the proteins to dissociate from each other [170]. In Study III, we used acid ethanol extraction to extract insulin and glucagon.

Immunoassays

In this thesis, we used two different types of immunoassays to measure insulin and glucagon. The first method was an ELISA that was used to quantify glucagon in α -TC1-6 cells. The method is based on a solid phase two-site immunoassay sandwich technique. Two monoclonal antibodies are directed against two different antigenic determinants on the target molecule (for example, glucagon). One of the antibodies is bound to microtitration wells. Upon adding the sample to the well, the target molecule will bind to the bound antibody; unbound molecules are then washed away, and the second antibody that is conjugated to peroxidase is added. Then a substrate is

added, absorbance is measured, and the concentration of the sample is determined using known concentrations from a standard curve [171, 172].

The second method was a solid-phase RIA that was used to quantify insulin from INS-1 832/13 β -cells, and to measure insulin and glucagon from human pancreatic islets. In this method, an anti-insulin antibody is bound to tubes. ^{125}I -labeled insulin and the sample of interest are added to the same tube, in which they compete to bind to the anti-insulin antibody. After an overnight incubation, all unbound material is decanted, and radioactivity is measured. Higher insulin concentrations will result in lower radioactivity. The concentration of insulin in the sample is then determined using a standard curve [173].

The crystal violet proliferation assay

In this method, cells are stained with crystal violet, which stains the DNA. Cells are then washed with water to remove excess dye and non-adherent dead cells. Methanol is used to extract the dye, and absorbance is measured at 600 nanometers (nm). The intensity of the crystal violet staining, or the absorbance, is proportional to the number of viable cells [174]. In Study III, we used the crystal violet proliferation assay to determine whether overexpression of *Cdkn1a* affects proliferation of INS-1 832/13 β -cells.

Western blot

Western blot is a method that is used to separate and identify proteins. In this method, a mixture of proteins, often prepared from cell lysates, is separated according to molecular weight through gel electrophoresis. Separated proteins are then transferred to a membrane. The protein of interest can then be detected, and quantified by specific antibodies [175]. The HA tag is a nine-amino acid peptide sequence present in the human influenza virus HA protein [176]. HA-tagged proteins can be recognized by an anti-HA antibody, and detected by a number of techniques, including Western blot, without the need for a specific antibody for the target protein. In Study III, we transfected HA-tagged cDNAs for *Cdkn1a*, *Sept9* and *Pde7b* into cells, and used an anti-HA antibody to verify the overexpression of genes at the protein level using Western blot.

Insulin exocytosis

In Study IV, we wanted to study the effect of decreased expression of *Exoc3l* on insulin exocytosis. We used RNA interference to post-transcriptionally silence *Exoc3l*

expression in INS-1 832/13 β -cells, and then measured insulin exocytosis using the patch-clamp technique.

RNA interference

RNA interference (RNAi) is an evolutionary conserved defense mechanism that is used by eukaryotic cells to protect them from invading viruses and transposons. Upon invasion by a foreign genome, and the synthesis of double stranded RNA (ds RNA), the RNAi machinery is triggered. An RNase III enzyme, known as Dicer, cleaves the dsRNA into small interfering RNAs (siRNAs). These siRNAs form duplexes that are then incorporated into the RNA-induced silencing complex (RISC), endonuclease. RISC then targets degradation of mRNAs that are homologous in sequence to the siRNA. Since its introduction, RNAi has been a valuable tool for studying gene function in mammalian cells. A gene of interest can be silenced post-transcriptionally by transfecting cells with synthetic siRNAs that are homologous to the sequence of its transcript, and the consequences of this silencing can then be studied [177].

Patch-clamp technique

In Study III, we used the perforated patch-clamp technique to measure β -cell exocytosis. In this method, a glass micropipette is attached to the cell membrane and a pore-forming antibiotic included in the intracellular solution within the micropipette will form pores in the sealed cell membrane patch (Figure 18A). The advantage of this configuration is that the intracellular organelles remain intact. Depolarization of the plasma membrane evokes electrical current and capacitance changes that can then be measured [178]. The fusion of an insulin granule membrane with the β -cell plasma membrane leads to increases in membrane size, and can be captured and measured as plasma membrane capacitance changes (Figure 18B) [179].

Glucagon secretion

In Study III, we wanted to test if α -cells overexpressing *Cdkn1a*, *Pde7b*, and *Sept9* secreted more glucagon than control cells. To do so, we transfected α -TC1-6 cells with pcDNA3.1 expression vectors containing cDNA for *Cdkn1a*, *Pde7b* or *Sept9*. After 48 hours of transfection, we performed a glucagon secretion assay.

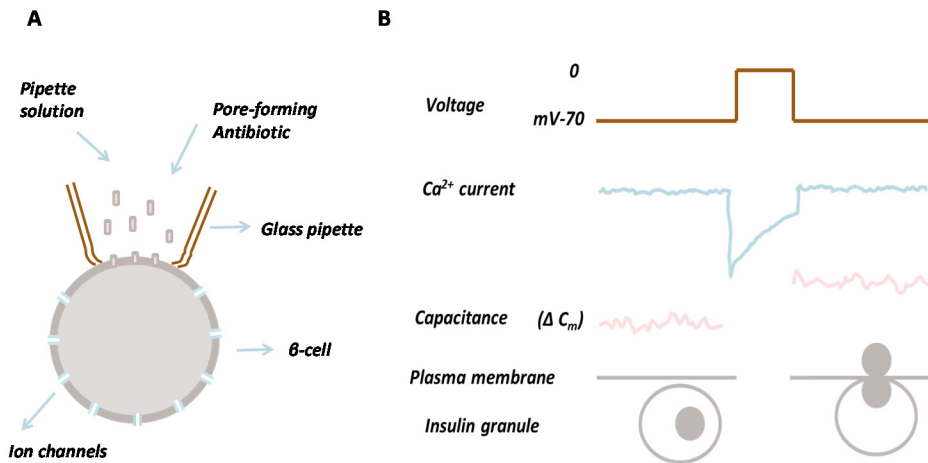


Figure 18. Measuring β -cell exocytosis using the perforated patch-clamp technique. Perforated patch-clamp configuration (A). Changes in membrane capacitance (ΔC_m) following depolarization-evoked exocytosis (B). Pictures are adapted from [180, 181].

Glucagon secretion assay

Prior to measuring glucagon secretion, we incubated cells in HBSS, supplemented with 5.5 mM glucose for two hours at 37°C. We then stimulated the α -cells to secrete glucagon by incubating them with HBSS supplemented with 1 or 16.7 mM glucose for one hour at 37°C. We collected the supernatant, and measured the glucagon level using an ELISA. We then lysed the α -cells with water, scraped them, and the lysate was collected and sonicated to stimulate the α -cells to release their content. We then used centrifugation to remove insoluble material, and measured the protein concentration of the supernatant using the BCA assay.

BCA assay

The bicinchoninic acid assay (BCA assay) is a biochemical assay that is used to determine the protein concentration of a solution. The basic principle behind the method is that protein peptide bonds reduce Cu^{2+} to Cu^+ ; the Cu^+ then forms a complex with bicinchoninic acid which results in a purple colored product that strongly absorbs light at 562 nm. The amount of absorbed light is proportional to the concentration of proteins in the solution [182].

In vitro methylation assay

We used *in vitro* methylation assays in Studies II and III to examine whether DNA methylation affects gene expression. We inserted promoter and/or enhancer sequences for *PDX-1*, and promoter sequences for *CDKN1A* and *PDE7b*, into the pCpGL luciferase expression vector. Unlike traditional vectors that contain many CpG sites in their backbone that can repress expression when methylated, the pCpGL vector does not contain CpG sites and hence only represses expression when the inserted promoter sequence is methylated [183]. The reporter gene constructs were then mock-methylated, or methylated with the methyltransferases SssI, that methylates all the CpG sites, or HhaI, that methylates the internal cytosine residue in a GCGC sequence only. Following *in vitro* methylation, we cotransfected cells with the pCpGL firefly luciferase construct containing the DNA sequence of interest, and a renilla luciferase construct was used as an internal control to minimize experimental variability. We measured the luciferase activity of the cell lysates obtained after 48 hours of transfection using the dual-luciferase assay that allows measurement of both firefly and renilla luciferase sequentially in the same sample. We normalized the firefly luciferase activity of each construct against renilla luciferase activity, and compared it with cells transfected with an empty construct.

Statistical Analysis

Data is described as the mean \pm standard deviation (SD) or the mean \pm standard error of mean (SEM). Comparisons between two independent groups were performed using a linear regression corrected for confounding factors, or a non-parametric Mann-Whitney test. Comparisons between three independent groups were performed using a Kruskal-Wallis H test. Comparisons between two dependent groups were calculated using a non-parametric Wilcoxon test. Correlations between two variables were calculated using Spearman or Pearson correlation coefficients. A PCA was used to identify possible sources of variation in genome-wide DNA methylation data. An FDR was used to correct for multiple testing. Chi-square tests were used to compare the observed number of differentially methylated probes in T2D islets with the expected number of probes on the 450k array. A logistic regression was used to examine if DNA methylation could predict future T2D.

Results

Studies I & II

Insulin promoter DNA methylation correlates negatively with insulin gene expression, and positively with HbA1c levels in human pancreatic islets.

Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes

Using a candidate gene approach, we wanted to determine whether DNA methylation of *INS* and *PDX-1* was altered in islets of subjects with T2D; and, if altered DNA methylation plays a role in impaired β -cell function in subjects with T2D. In Studies I and II, we analyzed DNA methylation and expression of both *INS* and *PDX-1* in pancreatic islets of nine T2D donors and up to 55 non-diabetic donors. *INS* and *PDX-1* mRNA expression, insulin content and GSIS were significantly reduced in T2D islets compared with non-diabetic islets (Figure 19B). In addition, *INS* mRNA levels correlated positively with insulin content, while *PDX-1* expression correlated positively with *INS* expression and GSIS. Both *INS* and *PDX-1* expression correlated negatively with HbA1c levels and BMI.

To examine if DNA methylation of *INS* and *PDX-1* was altered in T2D islets, we analyzed DNA methylation at 25 CpG sites in the *INS* promoter, and a total of 35 CpG sites in the *PDX-1* promoter and enhancer regions using EpiTYPER and pyrosequencing in human pancreatic islets. We found that four CpG sites surrounding the *INS* TSS, three CpG sites in the *PDX-1* distal promoter, and seven CpG sites in the *PDX-1* enhancer, showed increased DNA methylation in T2D islets compared with non-diabetic islets (Figure 19A). The absolute increase in DNA methylation in T2D islets ranged from 4.4 to 18 percent.

We then proceeded to test whether expression of *INS* and *PDX-1* was associated with DNA methylation. We found that DNA methylation at both *INS* promoter and *PDX-1* promoter and enhancer regions correlated negatively with their gene expression in human pancreatic islets. The strongest correlations for *PDX-1* were found at the *PDX-1* enhancer region. Furthermore, *in vitro* methylation of human

PDX-1 promoter and enhancer regions suppressed reporter gene expression in INS-1 832/13 β -cells.

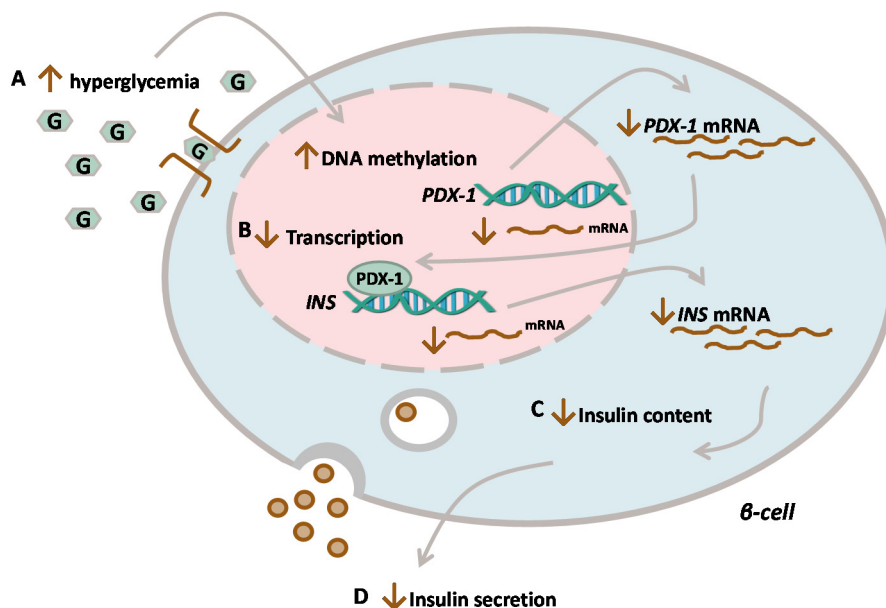


Figure 19. Increased DNA methylation and decreased expression of *INS* and *PDX-1* in T2D islets with impaired insulin secretion.

Hyperglycemia was associated with increased DNA methylation of *INS* and *PDX-1* in human pancreatic islets, and in INS-1 832/12 β -cells exposed to hyperglycemia (A). Increased DNA methylation correlated with reduced expression of both *INS* and *PDX-1* in human T2D islets, and suppressed reporter gene expression in INS-1 832/13 β -cells (B). Insulin content and GSIS were both reduced in human T2D islets (C and D).

Interestingly, we found that DNA methylation at both *INS* and *PDX-1* correlated positively with HbA1c levels in human pancreatic islet donors. To further examine if hyperglycemia affects DNA methylation in β -cells, we cultured INS-1 832/13 β -cells in high glucose for 72 hours, and found that hyperglycemia increased DNA methylation of both *Ins* and *Pdx-1* promoters. In addition *Pdx-1* expression decreased, and *Dnmt1* expression increased, in INS-1 832/13 β -cells exposed to hyperglycemia.

Finally, to examine if DNA methylation may play a role in β -cell specific expression of *INS* and *PDX-1*, we compared DNA methylation of human FACS-sorted β - and α -cells, and found that DNA methylation was lower in human β -cells.

Study III

Genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors identifies candidate genes that influence insulin secretion.

To follow up on our findings from Studies I and II, we analyzed DNA methylation of approximately 480,000 sites in human pancreatic islets from 15 subjects with T2D, and 34 non-diabetic donors, using the Infinium HumanMethylation450 BeadChip. Our aim was to further examine the role of epigenetics in the pathogenesis of T2D by using a genome-wide approach, and to describe the human pancreatic islet methylome. We identified differentially methylated sites using linear regression, correcting for age, gender, BMI, islet purity, islet days in culture and batch; and we used a FDR to correct for multiple testing.

We found that CpG sites located in genomic regions close to the TSS showed a relatively low degree of DNA methylation compared with those further away (Figures 20A and 20B). However, we did not find any difference in the average degree of DNA methylation of genomic regions between T2D and non-diabetic islets, suggesting that the changes are gene-specific rather than global.

Next, we proceeded to compare the methylation of individual CpG sites, and identified 1,649 CpG sites (853 genes) with altered DNA methylation in T2D islets ($\Delta \geq 5\%$ and $Q\text{-value} < 0.05$). The majority (97 percent) of these CpG sites showed decreased DNA methylation in T2D islets (Figure 20C). Approximately 60 percent of differentially methylated sites were located in the open sea (not annotated to any CpG island), while approximately 34 percent were located in gene bodies (Figures 20A and 20B). Differentially methylated CpG sites were overrepresented in the open sea and intergenic regions (not annotated to any gene), while they were underrepresented in the TSS200, 1st exon and CpG islands (Figures 20A and 20B). Interestingly, we found that some of the T2D candidate genes, including: *TCF7L2*, *FTO* and *KCNQ1*, were among the differentially methylated CpG sites in T2D islets.

102 out of 853 of the differentially methylated genes were also differentially expressed in T2D islets. 75 percent of the differentially methylated and expressed genes had an inverse relationship (Figure 20D). Furthermore, *in vitro* methylation of human *CDKN1A* and *PDE7B* promoters suppressed reporter gene expression.

T2D islets showed a decrease in DNA methylation of *CDKN1A*, *PDE7B* and *SEPT9*, with a simultaneous increase in their mRNA expression (Figure 20E). Overexpression of *Cdkn1a*, *Pde7b* and *Sept9* perturbed insulin and glucagon secretion in clonal β - and α - cells (Figure 20E). Additionally, T2D islets showed an increase in *EXOC3L2* DNA methylation, and a decrease in its expression. Furthermore, silencing of *Exoc3l2* in INS-1 832/13 β -cells reduced insulin exocytosis (Figure 20E).

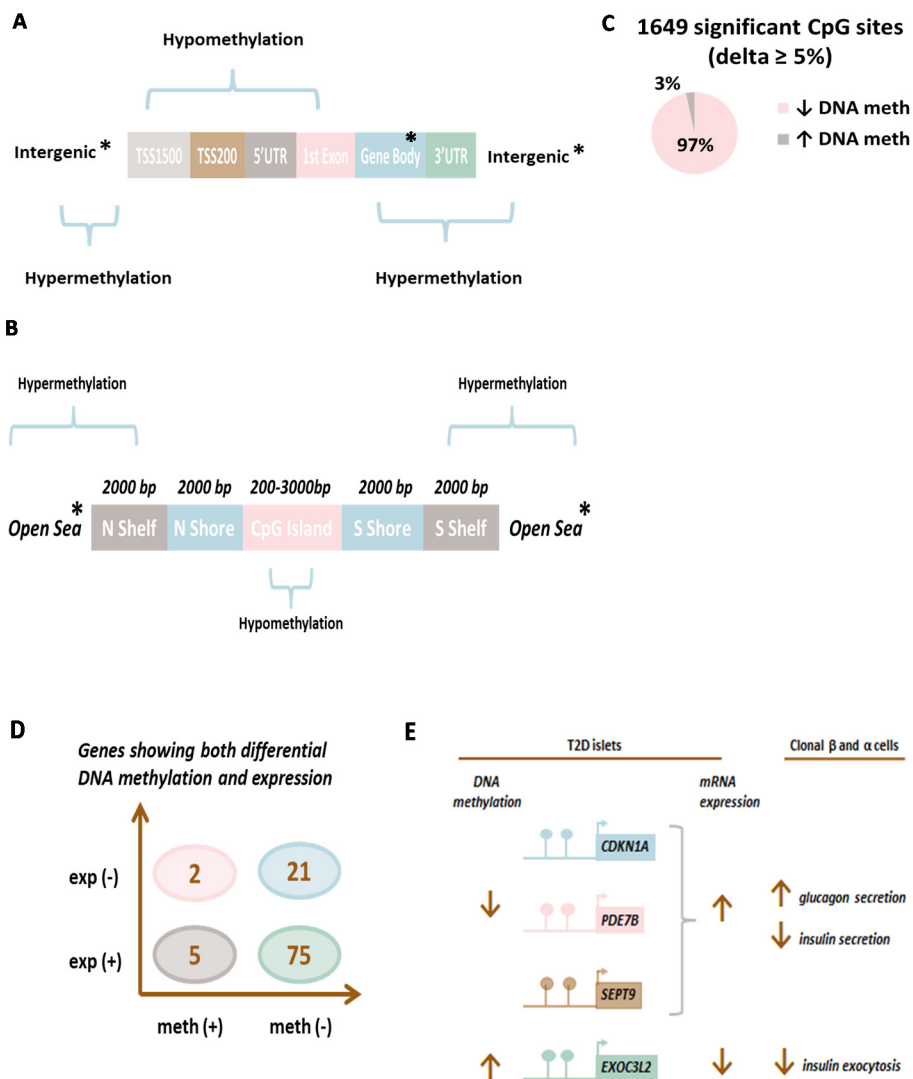


Figure 20. Genome-wide DNA methylation analysis of human pancreatic islets.

Genomic regions of hypo- and hypermethylation in human pancreatic islets (A-B). * Genomic regions containing the majority of differentially methylated CpG sites in T2D islets. Percent of CpG sites showing an increase or decrease in DNA methylation in T2D islets (C). Number of genes showing both differential DNA methylation and gene expression in human pancreatic islets (D). *In vitro* manipulation of differentially methylated and expressed genes in β - and α - cell lines causes impaired insulin exocytosis and secretion and impaired glucagon secretion (E)

Study IV

Identification of CpG-SNPs associated with type 2 diabetes, and differential DNA methylation in human pancreatic islets.

CpG-SNPs are SNPs that can introduce or remove a CpG site [184]. CpG-SNPs can affect gene function via a number of molecular mechanisms, depending on their genomic location [75, 85, 185-187]. The aim of this study was to examine if any of 40 SNPs associated with T2D are CpG-SNPs, and if they may be associated with differential DNA methylation in human pancreatic islets from 84 non-diabetic donors.

We found that 19 out of 40 (48 percent) of T2D-associated SNPs were CpG-SNPs (Figure 21A). Among these CpG-SNPs were SNPs annotated to *TCF7L2*, *KCNQ1*, *CDKAL1* and *SLC30A8*. All 16 analyzed CpG-SNPs were associated with differential DNA methylation at the CpG site directly affected by the SNP in human pancreatic islets (Figure 21B). In some cases, the CpG-SNP was also associated with differential DNA methylation of surrounding CpG sites, for example, *TCF7L2*, *KCNQ1* and *SLC30A8*.

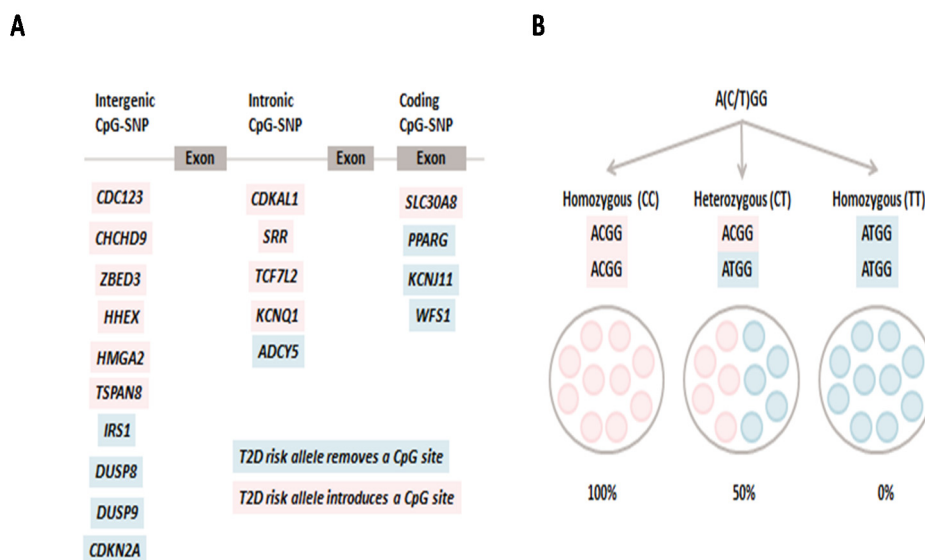


Figure 21. T2D associated CpG-SNPs and differential DNA methylation.

A. 19 out of 40 T2D associated SNPs introduce (n = 11) or remove (n = 8) a CpG site. Ten of the T2D CpG-SNPs were intergenic, while 9 were intragenic (5 intronic and 4 exonic). B. Differential DNA

methylation at the rs7901695 (*TCF7L2*) CpG-SNP. Individuals who were homozygous for the C risk allele (introduces a CpG site) showed nearly 100 percent methylation at that CpG site, in contrast to individuals that were homozygous for the non-risk allele (removes a CpG site) who showed nearly 0 percent methylation.

Since DNA methylation has been associated with alternative splicing events [85], we wanted to test if any of the nine T2D-associated CpG-SNPs located in introns or exons are associated with differentially expressed exons, as assessed by the splicing index. We found that CpG-SNPs located in *SLC30A8*, *WFS1*, *CDKAL1* and *TCF7L2* were associated with the expression of specific exons in the respective gene in human pancreatic islets.

We further wanted to examine if risk alleles of the associated T2D CpG-SNPs were associated with lower insulin content and/or secretion, or elevated glucagon secretion. We found that CpG-SNPs annotated to *ADCY5* and *HHEX* were associated with lower insulin secretion, while CpG SNPs annotated to *ADCY5* and *KCNQ1* were associated with elevated glucagon secretion. In addition, rs564398 (*CDKN2A*) was associated with lower insulin content.

Moreover, the identified T2D associated CpG-SNPs were in strong LD with 295 SNPs, including 91 CpG-SNPs. We analyzed DNA methylation in three CpG-SNPs that were in strong LD with T2D associated CpG-SNPs annotated to *CDKAL1*, *WFS1* and *HHEX*, and found that they were all associated with differential DNA methylation in human pancreatic islets.

Study V

DNA methylation at ABCG1 and PHOSHO1 in blood DNA is associated with future type 2 diabetes risk.

Identification of subjects with a high risk of developing T2D is fundamental for disease prevention. The aim of this study was to examine if five recently reported DNA methylation loci in blood DNA could predict future T2D in subjects from the Botnia prospective study, and if their DNA methylation is altered in target tissues for T2D.

Using pyrosequencing, we analyzed DNA methylation at cg06500161 (*ABCG1*), cg02650017 (*PHOSPHO1*), cg18181703 (*SOCS3*), cg11024682 (*SREBF1*) and cg19693031 (*TXNIP*) in baseline blood DNA from 258 non-diabetic subjects from the Botnia prospective study. After an average follow up of 8.1 ± 3.7 years, 129 subjects of the 258 had developed T2D.

We found that DNA methylation at the *ABCG1* locus cg06500161 in blood DNA was associated with an increased risk for future T2D (Odds ratio (OR) = 1.09, 95% confidence interval (CI) = 1.02–1.16, P value = 0.007) after adjustment for age, gender, fasting glucose, and family relation. Furthermore, the level of DNA methylation at the *ABCG1* locus cg06500161 in blood DNA correlated positively with BMI, HbA1c levels, fasting insulin and triglyceride levels, and was increased in adipose tissue and blood from the diabetic twin among monozygotic twin pairs discordant for T2D (Figure 22).

On the other hand, DNA methylation at the *PHOSPHO1* locus cg02650017 in blood DNA was associated with a decreased risk for future T2D (OR = 0.85, 95% CI = 0.75–0.95, P value = 0.006) after adjustment for age, gender, fasting glucose, and family relation. In addition, DNA methylation at the *PHOSPHO1* locus cg02650017 in blood correlated positively with high-density lipoprotein (HDL) levels, and was decreased in skeletal muscle from the diabetic twin among monozygotic twin pairs discordant for T2D (Figure 22).

We also found that DNA methylation at a number of surrounding CpG sites in these five genes was altered in human pancreatic islets, liver, adipose tissue, skeletal muscle and blood from subjects with T2D compared with controls.

To further investigate if the effect of altered DNA methylation on the phenotype could be mediated by altered gene expression, we correlated DNA methylation at the five CpG sites with gene expression from the same tissue and individual. DNA methylation at cg18181703 correlated positively with *SOCS3* expression in human pancreatic islets, and negatively with *SOCS3* expression in adipose tissue.

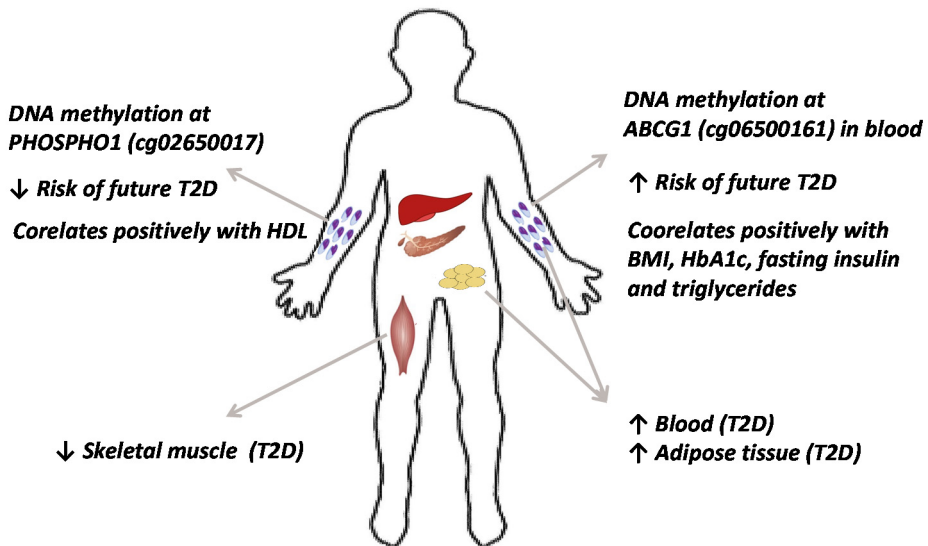


Figure 22. DNA methylation at *ABCG1* and *PHOSPHO1* loci is associated with future T2D risk. DNA methylation at both the *ABCG1* and *PHOSPHO1* locus is associated with a number of T2D risk factors and dysregulated in T2D target tissues in subjects with T2D.

Additionally, expression of both *ABCG1* and *TXNIP* was altered in skeletal muscle from T2D subjects, while expression of *SREBF1* was altered in liver and skeletal muscle from subjects with T2D.

Finally, we wanted to test if DNA methylation in blood could be used as a surrogate marker for DNA methylation in target tissues for T2D. We found that DNA methylation at cg18181703 (*SOCS3*) and cg11024682 (*SREBF1*) in blood correlated with their DNA methylation in adipose tissue from the same individual (Regression coefficients = 0.31 and 0.40, *P* values = 0.010 and 0.052, *N* = 28).

Discussion

Islet dysfunction is central to the development and progression of T2D [4]. T2D is a bi-hormonal disease characterized by impaired insulin and glucagon secretion [9]. For successful secretion of insulin, β -cells need to perform a number of functions, including: synthesizing insulin and making sure that it is properly folded, cleaved and packaged into granules; preparing the granules so that they are ready to be released; sensing changes in the extracellular glucose levels; taking up glucose, and metabolizing it; and releasing insulin granules when they are needed via the exocytosis machinery [1]. Evidence from human, rodent and cell line models have reported defects in many of these steps in T2D and its associated phenotypes. This demonstrates that impaired β -cell function can be caused by different mechanisms acting in isolation, or in combination. It should be stressed that some mechanisms may initiate the disease, while others are more likely to play a role in disease progression [1, 4].

Consistent with previous studies, we found that isolated islets from subjects with T2D synthesize and secrete less insulin. Furthermore, we also found that expression of the β -cell specific TF *PDX-1* was reduced in T2D islets, and that its expression correlated positively with insulin expression and GSIS. PDX-1 plays a key role in maintaining β -cell identity by positively regulating insulin and islet amyloid polypeptide expression, while repressing glucagon expression [38, 42]. Our findings support previous studies showing that decreased expression of *PDX-1* results in impaired insulin secretion [42, 188, 189]. It has also been suggested that changes in gene and protein expression of β -cell-specific genes may play a role in β -cell dedifferentiation or loss of phenotype in T2D [6, 92].

Epigenetic modifications regulate the expression of both *INS* and *PDX-1*, exclusively in pancreatic β -cells [96, 98, 190, 191]. In support of this, we found that DNA methylation of the *INS* and *PDX-1* promoters was higher in blood and α -cells than β -cells. Importantly, we showed for the first time that DNA methylation of cis-acting regulatory elements for both *INS* and *PDX-1* is increased in human islets from subjects with T2D simultaneously with their reduced mRNA expression. Our *in vitro* methylation experiments demonstrate that altered DNA methylation of certain genomic regions (for example, *PDX-1* enhancer) may have a more pronounced effect on gene expression than other regions. Similarly, it has been shown that *in vitro* methylation of a single CpG site in the *INS* promoter suppressed reporter gene activity by 50% [190]. Interestingly, CpG sites located in the *PDX-1* enhancer region displayed the largest increase in DNA methylation in T2D islets, and the strongest

correlations with *PDX-1* expression, indicating that epigenetic regulation of this region is associated with clinical outcomes.

In line with previous studies demonstrating that hyperglycemia may induce persistent epigenetic changes in vascular cells that alter the expression of pro-inflammatory genes [192], we found that DNA methylation levels correlated positively with HbA1c levels in human pancreatic islets. Additionally, DNA methylation of *Ins* and *Pdx-1* was increased in clonal rat β -cells exposed to hyperglycemia, which was associated with decreased expression of *Pdx-1*, but increased *Dnmt1* expression. Although cause and consequence are still debated, studies in rodents have elegantly shown that exposure to adverse environmental factors at an early developmental stage can induce epigenetic changes that can persist till adulthood and cause disease [96, 97, 193]. Importantly, it has been shown that rats exposed to an adverse intrauterine environment exhibit an 80 percent reduction in *Pdx-1* expression in adulthood, which was associated with a number of epigenetic changes at the *Pdx-1* proximal promoter, including an increase in DNA methylation [96]. Furthermore, DNA methylation in human β -cells plays an essential role in enabling the coupling of insulin secretion to glucose levels in the first five years of life [87]. Similar to the pathogenesis of cancer, it may be that T2D results from a two-hit process, where some epigenetic changes are induced at an early stage and are involved in initiating diabetes, while others are induced later by hyperglycemia, accelerating disease progression and β -cell dysfunction.

Prior to the emergence of epigenome-wide tools, DNA methylation studies focused mainly on promoter regions. However, epigenome-wide tools, such as the 450k array, have enabled us to investigate methylation in other genomic contexts. In general, in human pancreatic islets and β - and α -cells, CpG sites that were located near the TSS showed a relatively low degree of DNA methylation compared with those further away. This probably creates a permissive chromatin state that allows various TFs and proteins to bind. However, the majority of CpG sites with altered DNA methylation in T2D islets were located in the gene body, or in regions not annotated to any gene or CpG islands, for example, the intergenic and open sea regions.

Our epigenome-wide analysis identified 3,116 CpG sites with altered DNA methylation in T2D islets. Out of which 1,649 CpG sites (853 genes) had absolute differences in DNA methylation $\geq 5\%$. These genes included genes with a known function in pancreatic islets, exocytosis and apoptosis, as well as many novel genes with no previous association with islets or T2D. In addition, 17 of the T2D candidate genes identified by GWAS were among the list of genes with altered DNA methylation in T2D islets, including *TCF7L2*, *THADA*, *KCNQ1*, *FTO* and *IRS1*. Notably, some of these genes have been implicated in β -cell function [57, 58].

Interestingly, although we observed an increase in DNA methylation of both *INS* and *PDX-1* in T2D islets, our epigenome-wide analysis showed that the majority of differentially methylated CpG sites in T2D islets have a decrease in DNA

methylation. Furthermore, we could not find any differences in the average degree of DNA methylation in the different genomic regions, suggesting that the changes were gene-specific rather than global. It is as yet unclear what makes the DNA methylation/demethylation machinery target certain parts of the genome but not others in response to environmental stimulus, but suggested mediators include DNA binding proteins or non-coding RNAs [74]. Interestingly, a recent study from our group reporting hypomethylation in liver from subjects with T2D also found that folate levels, which are a source of methyl groups, were reduced in subjects with T2D [134]. Moreover, metabolism of folate and the synthesis of the methyl donor, S-adenosylmethionine (SAM), require ATP. ATP is generated by glycolysis and oxidative phosphorylation, which is impaired in T2D islets [194, 195]. Additionally, offspring of pregnant Agouti mice that were fed a control diet, developed obesity and diabetes in adulthood, in contrast to the offspring of pregnant Agouti mice that were given a diet supplemented with methyl donors [193]. Another possible mechanism could be altered expression/activity of enzymes, such as DNMTs and TETs, which play a role in the methylation/demethylation machinery.

Although, DNA methylation has been generally associated with gene silencing, it has become clearer that the relationship between DNA methylation and expression is complex, and varies with genomic context [196]. It is not surprising, therefore, that only 102 out of 853 of the differentially methylated genes were also differentially expressed in T2D islets, with the majority showing decreased DNA methylation and increased expression. The lack of association between DNA methylation and gene expression for some CpG sites may point towards the different roles of DNA methylation based on genomic context. For example, intergenic DNA methylation plays a role in genomic stability, while gene body methylation has been shown to be both positively and negatively associated with gene expression [78, 83].

Our functional experiments provide further evidence that altering the expression of *CDKN1A*, *PDE7B* and *EXOC3L2* simulating the situation in T2D results in impaired insulin exocytosis, secretion and impaired glucagon secretion in cell line models. *CDKN1A* is a tumor suppressor and negative regulator of the cell cycle, *PDE7B* is a cyclic adenosine monophosphate-specific (cAMP-specific) phosphodiesterase that regulates cAMP levels, and *EXOC3L2* is a member of the exocyst complex [197-199]. Furthermore, *in vitro* methylation of the human *CDKN1A* and *PDE7B* promoters suppressed reporter gene activity in clonal β -cells, providing evidence of an association between DNA methylation and gene expression levels in these genes. These results support the hypothesis that altered DNA methylation in T2D islets may alter the expression of certain genes that then contribute to islet dysfunction.

To date, more than 60 genetic variants have been associated with T2D [56]. While the majority have been associated with β -cell function, it is still not clear how they exert their function, especially since many of them are non-coding SNPs [57]. Interestingly, we found that nearly half of the T2D-associated SNPs were CpG-SNPs

that introduce or remove a CpG site. Furthermore, some of the CpG-SNPs were associated with altered gene expression, splicing and hormonal secretion in human pancreatic islets, suggesting that some of the T2D SNPs may act through epigenetic mechanisms to alter expression and protein function. Subsequently, other studies have shown that a number of the T2D-associated non-coding variants are located in putative regulatory elements, including some that had enhancer activity [63-65]. It is worth mentioning that nearly 30% of point mutations that cause human genetic disease are CpG SNPs [81]. Consequently, individuals with different genotypes can display distinct responses to the same environmental exposure through acquiring different epigenetic modifications. Interestingly, this could potentially be an indirect mechanism for inheriting epigenetic modifications. Importantly, we showed that differential DNA methylation was not limited to the T2D-associated CpG-SNPs, but could also affect other CpG sites that were in LD, resulting in a more profound regional change to the chromatin structure.

In Study V, we confirmed the recently reported association between DNA methylation at two CpG sites in blood DNA, and future T2D in subjects from the Botnia prospective study. DNA methylation at cg06500161 (*ABCG1*) was associated with increased future T2D risk, BMI, HbA1c, fasting insulin, and triglyceride levels. Our findings are in line with other studies reporting associations between methylation at the same *ABCG1* locus, and a number of T2D risk factors, including: high circulating triglycerides, risk of myocardial infarction, fasting insulin, homeostasis model assessment- estimated insulin resistance (HOMA-IR), and decreased HDL levels [119, 200-202]. *ABCG1* is a cholesterol transporter, and plays an important role in maintaining cholesterol homeostasis by removing excess cholesterol from peripheral tissues, and transporting it to the liver. Accumulation of cholesterol in islets compromises β -cell function, and reduces insulin secretion. Indeed, loss of both *ABCG1* and *ABCA1* (another cholesterol transporter from the same family) in mice results in impaired insulin secretion, increased fasting glucose levels, and inflammation of pancreatic β -cells [203, 204]. Interestingly, DNA methylation at the *ABCG1* locus was increased in adipose tissue and blood from subjects with T2D.

Additionally, we found that DNA methylation at the cg02650017 (*PHOSPHO1*) locus in blood DNA was associated with decreased risk of T2D, and increased HDL levels. *PHOSPHO1* is a phosphatase that plays a role in skeletal mineralization, but has also been implicated in vascular mineralization [205, 206]. Hypercholesterolemia, aging and diabetes often lead to cardiovascular calcification [207]. *PHOSPHO1* is an attractive target for cardiovascular therapy [206]. Importantly, we found that DNA methylation at the *PHOSPHO1* locus was decreased in skeletal muscle from subjects with T2D.

A novel finding from Study V, was that for some CpG sites, DNA methylation was altered in different tissues from the same individual, and that DNA methylation at some CpG sites correlated in tissues from the same individual. Furthermore, a number of surrounding CpG sites also showed altered DNA methylation in T2D

target tissues, suggesting that the changes were regional and may be mediated by so-called metastable epi-alleles. Metastable epi-alleles arise from epigenetic modifications that are established during early development, and can result in differential expression of alleles from genetically identical individuals, or even from different tissues from the same individual. Despite their stochastic nature, metastable epi-alleles can potentially be inherited trans-generationally, can be altered by the environment, and can affect all tissues of the body [115].

Taken together, our studies suggest that epigenetic dysregulation in the form of DNA methylation is present in islets from subjects with T2D, and can contribute to islet dysfunction. Furthermore, epigenetic dysregulation may be induced by environmental factors, including hyperglycemia, and is influenced by genetic variation. Consequently, this may contribute to the heterogeneity of T2D. The plasticity of the epigenome makes it a good target for T2D therapy, and DNA methylation biomarkers could potentially be used to identify individuals with a high risk of developing T2D in the future.

Summary and general conclusions

Epigenetic dysregulation of genes involved in maintaining β -cell identity and function may contribute to islet dysfunction observed in subjects with T2D. Though it is difficult to distinguish the cause from consequence, our findings suggest that hyperglycemia may induce changes in DNA methylation in human pancreatic islets.

Epigenetic dysregulation in T2D islets is non-randomly spread throughout the genome, and can alter gene activity in a context-dependent manner. Among the list of epigenetically dysregulated genes were those known to be associated with T2D. Additionally, we identified new target genes with altered DNA methylation and expression, including some with a role in insulin and glucagon secretion.

Epigenetic variation in human pancreatic islets is influenced by genetic variation, and can mediate its effects on islet function via a number of mechanisms, including altering gene expression and alternative splicing.

DNA methylation at two markers in blood DNA from non-diabetic individuals was associated with increased risk of future T2D, and T2D risk factors. Altered DNA methylation of the same CpG site/gene in multiple tissues from the same individual may be induced by exposure to environmental cues at an early developmental stage.

We provide a reference methylome for human pancreatic islets in health and disease. The next step would be to study the epigenome for different islet cell types from both healthy and subjects with T2D. This information will be useful for current attempts to generate functional β -cells, to induce β -cell proliferation, and to preserve or enhance islet function via epigenetic drugs.

Popular science summary

Sugar is the main source of energy in our bodies. If your blood sugar drops below a certain level, it can cause serious damage to your brain. On the other hand, having a very high blood sugar level for a long time can cause diabetes. For this reason, blood sugar levels in our bodies are maintained within a certain range by a group of hormones that work together. These hormones are produced by the pancreatic islets that consist of five cell types. Each cell type produces a special hormone that plays a role in the fine-tuning of sugar levels in our bodies. When you have a meal, your blood sugar will rise, and the pancreatic beta-cells will sense this and release insulin. Insulin is a hormone that sends a signal to certain cells in your body to store excess sugar and remove it from blood. When you haven't eaten for some time, your blood sugar goes down and pancreatic alpha-cells produce glucagon. Glucagon does the opposite of insulin and sends a signal to certain cells in your body to release some sugar from their stores. Patients with type 2 diabetes have problems producing enough insulin, and produce more glucagon than is needed. To make things worse, the cells that are supposed to receive signals from insulin to store sugar are not very responsive. As a result, sugar is not cleared from your blood. Having high blood sugar for a long time is one of the main reasons for heart attacks, kidney failure, blindness and lower limb amputation.

Why do certain people get type 2 diabetes? Many factors can increase your risk of getting type 2 diabetes. Some are genetic (determined by the genes you inherit from your parents), and others are environmental. People that have diabetes running in their family are more likely to become diabetic themselves. The main reasons why the number of people with type 2 diabetes is increasing worldwide are probably: being overweight, eating unhealthy food, and not exercising. Other factors include: smoking, ethnicity, aging, high blood pressure, and poor nutrition during pregnancy. One way in which these different environmental factors can affect the way your body works is via epigenetic modifications.

What are epigenetic modifications, and how do they work? Epigenetic modifications are molecules that are attached to your DNA and can change the way your genes behave without changing the gene itself. The DNA that you inherit from your parents is made up of a sequence of four letters (A, T, C and G); this sequence provides your cells with an instruction manual that tells them what to do. Although all the cells in your body carry exactly the same instruction manual (DNA), they look and behave very differently. This is because a group of proteins and small molecules

that are attached to your DNA make every cell-type use different parts of the instruction manual (express different genes). In other words, each cell has a different epigenome on top of the DNA that tells it to express a group of genes, and prevents it from expressing others. The epigenome can help your genome remember certain experiences that it was exposed to. They could be good experiences (for example, healthy diet and exercise) that help your body adapt, or they could be bad experiences (for example, unhealthy diet, pollutants, starvation and poor nutrition as a fetus) that make you sick. There are different types of epigenetic modifications that give different signals, and can be located in different parts of the DNA sequence. DNA methylation is one of these epigenetic modifications; it is made by the attachment of a molecule, known as a methyl group, to the letter C in your DNA sequence (mainly those followed by the letter G). These methyl groups can block certain proteins from reaching the switch button that turns your genes on.

DNA methylation of pancreatic islets is different in patients with type 2 diabetes. Pancreatic islets from patients with type 2 diabetes have either more or fewer of these methyl groups in different parts of their DNA sequence than healthy people. In general, patients with type 2 diabetes have fewer methyl groups attached to their DNA, especially in certain parts of the DNA sequence. In some cases, the genes that are located in these regions behave differently in patients with type 2 diabetes, including genes that are important for the normal function of pancreatic islets and their ability to produce insulin and glucagon. For example, pancreatic islets from patients with type 2 diabetes have more methyl groups attached to the gene that produces insulin and another gene that regulates it. It is possible that these epigenetic changes are one of the reasons that make patients with type 2 diabetes produce less insulin.

The sequence of letters that makes up your DNA is predetermined by your parents. Because the methyl group is mainly added to the letter C, followed by a G, people that don't have CG cannot have a methyl group attached to their DNA. That means that many of the changes caused by the addition or removal of these methyl groups will be different for different people based on the sequence of letters that make up their DNA. If this happens in a region that plays a role in diabetes, people with a certain sequence of letters will be more affected than others.

Almost 50 percent of people are unaware that they have diabetes. By the time they are diagnosed, a lot of the damage has already been done to their bodies. Researchers have been trying to find a 'fingerprint' that can help them identify individuals that are likely to get type 2 diabetes, so that they can get help, and control their blood sugar level before it gets worse. A number of DNA 'fingerprints' have been found, but researchers are still trying to find even more reliable ones. Epigenetic 'fingerprints' are an interesting tool that is already being used in cancer research. We and other researchers are trying to find an epigenetic 'fingerprint' in blood DNA that could predict if an individual will get diabetes in the future.

How can I avoid becoming diabetic? You can avoid getting type 2 diabetes by exercising, eating healthy food, and avoiding many of the other risk factors. Although you can't change your genes, having a healthy lifestyle can protect you from getting diabetes, or at least delay its onset. We believe that sometimes an unhealthy lifestyle can make certain epigenetic changes to the pancreatic islets, and that these changes can cause diabetes. The good news is that some of these changes can be reversed by a healthy lifestyle, and thus protect you from getting diabetes.

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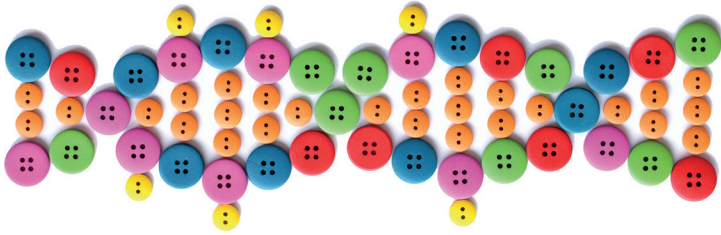
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Methyl groups in yellow attached to DNA.

